TAMPERE UNIVERSITY OF TECHNOLOGY

Maria Vasilenko 223901

Feasibility of using biosensors for heavy metal detection in complex matrices such as bio-slurries.

Master of Science Thesis

Examiners: Professor Matti Karp
Professor Raghida Lepistö
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Abstract

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The quality of bioslurries that are used in industrial production and agriculture need to be watched very closely to avoid spreading of contaminants on area and poisoning of humans and animals. Because heavy metals are very stable and toxic in many chemical compositions, their amount should be estimated very thoroughly. A new approach that involved biosensors was tested in this study. Because the slurries are complex non-unified matrices which composed of two phases – solid and liquid, the cell behavior can varies a lot from the one that explained in water and so the estimation of ion concentration can be not reliable.

It was shown that the cell actually behave different in the slurries. Normally the dissolved compounds suppress the biosensor activity and, in the same time, the ions in the particles can released during the tests and interfere with the signal. So the concentration and the pretreatment of the samples should be chosen for every particular biosensor.

Additionally, there was an attempt to measure the heavy metal amount and to compare it with the results that were obtained on AAS. The data declares that the bioavailability may differ in the matrices and so the signals of biosensors vary even between the samples with the same total heavy metal amount.

Preface

The author would like to express her gratitude to Adjunct Professor Doctor **Raghida Lepistö** and to Professor Doctor **Matti Karp** for their guiding, critique, and mentorship throughout experiments and writing process. The author wishes to thank them for their valuable ideas and inspiration.

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Abbreviation

AAS – Atomic Absorption Spectroscopy
AMP – Adenosone Monophosphate
AN – Ammonium Nitrogen
AS – Activated Sludge
ATP –Adenosine Triphosphate
CAT – Chloramphenicol AcetylTransferase
CS-AAS – Continuous Source-Atomic Absorption Spectroscopy
DMPS – 2,3-Dimercapto-1-propanesulfonic acid
DMSA – Dimercaptosuccinic acid
EC – Electrical conductivity
EDTA – Ethylenediaminetetraacetic acid
ELISA – Enzyme-linked immunosorbent assay
GCL – Geosynthetic Clay Linen
HMM – Heavy metal medium
HPLC – High Pressure Liquid Chromatograph
IF – Induction Factor
LA – Luria Agar media
Lead sensor – *Pseudomonas putida* K2431.2440 pDNPczclux1
LB – Luria Broth media
LS-AAS – Line Source-Atomic Absorption Spectroscopy
MAP – Manganese Ammonium Phosphate
Mercury sensor - *E.coli* MC1061 pmerRBlux
MQ – Double distilled water
mRNA – matriceRNA
TCC – Thermochemical Conversion
TKN – Total Nitrogen
TS – Total Solids
TVS – Total Volatile Solids

1.0 Introduction

In this study there are two important issues of contemporary world meet – first, it is a need of fertilization material for agricultural industry and evaluation of its quality, and second, it is a testing and a describing the activity of the whole-cell biosensors that are one of the most interesting current approaches in the detecting of environment pollutants.

The interest to agricultural studies has been become stronger for the last decades due to increasing of world population and so growth of food consumption by people and animals. The industry dictates to find an option to increase a harvest without taking extra land. Therefore, the fertilization mechanisms are exposed in larger scale and the quality of the fertilizing material turns to be more and more important.

The convenient methods of the chemical detection of pollutants are rather expensive, require vast amount of time and trained personnel. In addition, they measure only the total amount of the chemicals without the differentiation on its bioavailability and non-bioavaliability, so they cannot sometimes be reliable in case of the high total concentration and the poor inclusion of the chemical in a food chain. For the more precise quality control there are some biological methods can come to help.

The biological methods involve different types of organisms from bacteria to, for instance, crustacean joint-legged which are used in a water quality monitoring. But bacteria can be most useful because of existence of various chemical transformation pathways and their small scale. So these organisms can be adapted for almost any scientific needs and transformed to high-throughput technology. For example, there are 6 different pathways of interaction with mercury as a heavy metal that cannot be included to compound synthesis inside a cell (Wood, 1984 in Boening, 2000) and so at least 6 different gene groups to use. Such situation suggests room to blow for sensor development. Furthermore, there are pathways and mechanisms for interacting to every other metal and all these ways can be adapted for experiments as well.

The first biosensor that was used in this project is designed with basis on *mer* operon and *luxCDABE* reporter complex and is established by Hakkila et al in 2002 but the first presentatio of using the similar sensing construct was in Selifonova et al, 1993. The biosensor is expecting to give a very good response evaluated in induction factor (IF) and to work both for organic and inorganic mercury compounds. And the second construct involves the sensor of bivalent metals based on cadmium operon *czc* and again on *luxCDABE* reporter. It was created by Hynninen et al in 2010 and adapted for Zn, Cd, Ni, and Pb.

The slurry samples were evaluated on concentration of the pollutants and there was a comparison of the data obtained with the biosensor and the more convenient method – AAS, in terms of sensibility, velocity and practical usability. It is expecting that the cells will give less response in biosensor measurement due to its reaction only to its bioavailable fraction.

The major tasks of this study are evaluation of the cell activity in complicated matrices like slurries, testing activity of the cells, evaluation of the conditions of their using, and also comparison of the obtained results with the ones that were got from AAS.

This report will be started with information about luminescence as a phenomenon and also some examples of it as a reporter gene will be provided. The current convenient physical method of heavy metals detecting in samples that are advised by international agencies – AAS – is described as well. Also there will be some experimental methods based on biological activity of organisms, including whole-cell receptors, and molecular in the following part. Heavy metals that are tested here will be described in the next part, data about their harmful effects and sites of appearance will be provided. The last part with be considered slurry, its production and sources of material.

The experimental part of the report will be started with the information about materials and methods that were used. There is also some data about the slurries that were tested in chemical way. And then the results, obtained with direct measurement on biosensors and discussion follow.

2.0 Theoretical Background

2.1 Luminescence

2.1.1 History of study of luminescence

Bioluminescence is a phenomenon that based in emitting of light by living objects during chemical reactions. It independently occurs several times in evolutions with different pathways involved. In nature, the luminescence can lure food or partners, provide communication, warn or treat surrounded, to scare or to distract, to camouflage on natural light sources (Fig. 1 and 2).

The light emission of living objects was appeared in literature and in essays of antic naturists for several times. Gaius Plinius Secundus in his Neturalis Historia described a glow of sea. But the systemic investigation of bioluminescence has been started in 1668 by Robert Boyle when he studied processes of burning and fluorescence of touchwood and found that both these processes are stopped in vacuum (anaerobic) environment. (Inge-Vechtomov, 2008)

The closer look was made by Rafael Dubois in 1887. He extracted the luminescented parts of *Pyrophorus* beetles, photophors, in water of different temperatures. He found that the extract emits light in cold water and does not in warm conditions. Moreover, after addition of warm extract to cold one which already had finished glowing, the both two portions start emiting light. He decided that is can be because of presence of heat resistant low mass compound and heat decomposed high mass protein part. And so the luminescence appears only in case of presence of both these fractions plus oxygen. The same results were got from *Pholas dactylus* mollusk photophor. This behavior is typical for enzymatical systems, so Dubois called the low mass fraction as luciferin and high mass as luciferase. (Harvey, 1957)

In 1920, Edmund Newton Harvey in Princeton found difference in two different systems of different organisms: luciferin of *Pholas sp* does not work with luciferase of crustacean *Cypridina* and vice versa. (Harvey, 1920 cited in Shimomura, 2006)

B. Bilter and W.D. McElroy in 1957 the firefly luciferase was extracted and defined as tiasol compound.

Osamu Simomura in late 1950s-early 1960s studied mechanism of luminescence in shrimp *Cypridina hilgendorfii* which was used as natural luminophor during Second World War by Japanese army – the dried crustacean had been started to glow after adding some water and gave enough light for reading messages. Dr Simomura managed to extract the luciferin in crystal phase. But later, he turned his studies to another object. In Princeton he studied a protein of jellyfish *Aequorea victoria*, and in comparison found that there are two different systems of light emission involved. The jellyfish's protein works in a completely other way unlike to classical two fractioned system of

luminescence. The further development leaded to creation of green fluorescent protein reporter system and a Nobel Prize to Simomura. (Pieribone, Gruber, 2005)

Figure 1. *Hawaiian bobtail cuttlefish (Euprymna scolopes). The blue light is luminescent algae which it uses for camouflage (Ormestad, 2010)*

Figure 2. *Japanese Fireflies (Luciola cruciate). Photo by H. Nomura (2008)*

2.1.2 Luminescence as a phenomenon

Bioluminescence is a particular case of chemiluminescence that appears in many reactions like recombination of free radicals or in red-ox reactions (white phosphorous in gases or oxidation of luminol in water etc). The light emission, in this instance, is a form of energy transformation that does not spread as heat but creates light – thus leads synthesis of a product in excited electron state. The production of light takes place only under two conditions: the produced energy is higher than ~41-71.5 kkal/mol (for luciferin) and the difference of energies of normal and excited product state was lower than the reaction enthalpy. Coherently, after transformation of the product from excited to normal state, there is a photon of a visible spectrum emits.

Quantum yield, or the ratio of the emitted electrons to total number of elementary reactions, of the great majority bioluminescence reactions is quite high – 0.88-0.1, unlike of the rest of chemiluminescence ones in the same pH conditions. It is caused by a presence of enzymes, so the processes are highly specific.

The wavelength of the emitted light depends on difference of energy in excited and normal state, while the half width of the emission band is usually~50nm. And because the process of transformation to excited state and back is reversible, the fluorescence spectra of oxidized form are close to bioluminescence spectrum: the process itself is still the same; the only difference is in method in moving of a molecule to excited state.

There are several independently occurred ways to create light in nature – for example, bacterial aldehyde-flavin system (*lux* genes), wormal aldehyde luciferins, tetrapirrole luciferins of D*inoflagellata* and some *Crustacea*, , imidapirazols of some marine animals, and luciferin (*luc* genes) of insects – made out of tiazol. (Shimomura, 2006) Two is the most studies are *lux* and *luc*: first is spread mostly in marine bacteria and it is so called a bacterial luciferase, and the second is a beetle or a firefly luciferase.

The maximum levels of molecule light emission in bioluminescence can be changed. For instance, oxiluciferin can vary it from 490-622 nm (green to red) with the same structure of the molecule. The variation can be between different species of beetles or even in one organism – for example, larvae of *Phrixothix sp.* shows presence of both red photophor on a head and yellowish green on a belly. It can be because of a presence of several forms of excited states and so different portions of added energy and different maximums of the spectras (Viviani et al, 1999).

The reason of presence of these different forms is that oxiluciferin can have some keto-enol tautomeric organization. So in the solution, there is always a mixture of ketonic and enolic tautomers. Theirs ratio depends on pH of the environment. In slightly base (pH 7.5-7.8) conditions the enol form dominates with spectra maximum at 587 nm (yellowish green light) while if the environment moves to acidic conditions (pH<6) the ketonic form appears mostly and the maximum in spectra moves to longer wavelength region with 618 nm (red region). After adding base to solution (pH>8), enol-anion oxiluciferin forms and the maximum moves to 556 nm. In the intermediate conditions, there is a mixture of enol and

ketonic forms that are composed by additive mix of ketonic and enolic molecules so the spectra is bimodal – have two high peaks. (Ugarova et al, 2005)

Another factor that effects on presence of different maximums is a microsurrounding of oxiluciferin in excited and normal states. On the energetic levels also energy of the contribution to solvent and number of hydrogen bonds effect. The more the excited molecule associated to its microsurrounding and the higher it is polarized, the lower energy of the excited state, the lower the energy of the emitted photon and the further the emission maximum moves to long waves region. (Nakatsu et al, 2006)

The third factor that affects on the excited state energy of the luciferin and so the spectral maximum is the relaxing processes in the solution. After the dissociation of a CO_2 from the 1,2-dioxiketane ancestor of oxiluciferin, there is a fast restructuration of the electron molecule and a rapid changing of its dipole moment, in the same time the excited molecule is still in the solvent net envelope of the previously been molecule. The life time of the excited luciferin molecule is about 10^{-9}-10^{-8} seconds. And if the solvent molecules or the surrounded protein chains are remain intact and do not reorganize in time to a new equilibrium state, the energy of the excited state is maximal and this maximum is in the short wavelength region. So the wavelength of the emitted light depends on a velocity of the relaxation of the microsurrounding – including the flexibility of the protein enzyme chains. (Ugarova et al, 2005)

As already mentioned above, the energy that is needed for light emission is ~41-71.5 kkal/mol, which correspond to the energy of the electromagnetic spectra in its visible part and also the energy portion is quite comparable to C-C bond of alkanes (~79 kkal/mol). This energy is much higher than the result energy of the most chemical reactions, even with macroergetic molecules. For instance, hydrolysis of ATP to AMP is 10.9 kkal/mol. Such energy can be reached only in case of single-stage reactions with a part of molecular or free radical forms of oxygen, so the vast majority of enzymes that convert luciferins are oxigenases (except some *Oligohaeta spp*. enzymes which are peroxidase-like). And of course, all the light emitting organisms are aerobic. (Ugarova et al, 2005)

Most of luciferins in oxidized state have cyclic strained intermediate peroxides-dioxitanons, where the angles of the 4 part cycle differ from the normal ones. These molecules dissociate with releasing of CO_2 and excited keton of luciferin. This mechanism is shown for luciferin of insects and celenterasins of marine animals. (Shimomura, 2006)

2.1.3 Luminescence reporter genes

Luminescence reporter proteins can be based of firefly and bacterial luciferases. Although both systems emit light their mechanisms differ.

Bacterial luciferases are found in *Vibrio fischeri* and in some *Photorabdis spp*. It oxidizes a reduced flavin mononucleotide and a fatty acid to a flavin mononucleotide and a carboxylic acid (see reaction 1). The figure 3 also shows the relation in wild type

bacteria, so in the marker cells in bioengineering there is only the left side on the picture saved while the right side of production of flavins is provided by the internal capacity of a cell.

$$FMNH_2 + RCHO + O_2 \text{ ----> } FMN + RCOOH + H_2O + \text{light (490nm)} \quad (1)$$

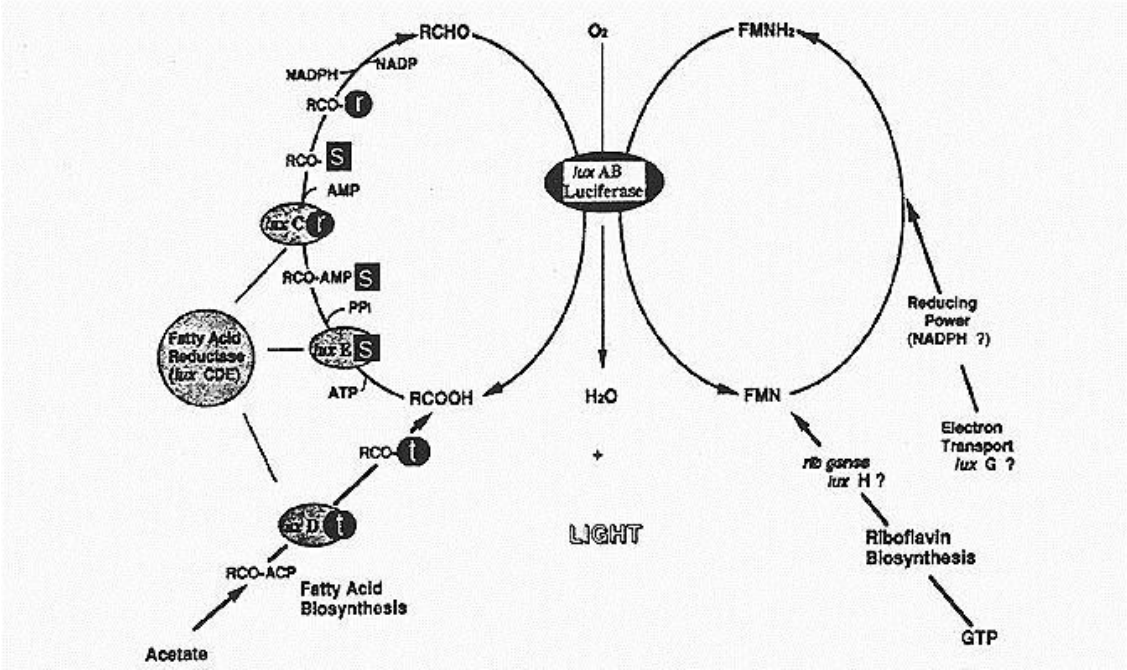

Figure 3*. Relation of the light emission cycle with the energy production cycle in wild-type cell. (Meighen, 1993)*

The process is aerobic with light emission at about 490 nm and a quantum yield ~0.1. Bacterial lucefirase by itself is coded by *luxAB,* but it also need 3 genes *luxCDE* that code aldehyde. The luciferase contains two subunits *a* and *b*. The second subunit, *b,* seems to increase the thermal stability of the system. The *luxCDE* genes on their order codes the reductases from fatty acides to aldehydes. If the construct has only *luxAB* it needs external aldehyde to be added. (Hakkila et al, 2002). The reaction is permanent and in case of *luxABCDE* needs no external control so it is very handy for experiments. In the native, wild type there are also some extra genes for regulation and receptoric parts, and some enzymes for flavin mononucleotide synthesis. Additionally, there can be a *luxY* gene, which codes YFP and can modulate kinetics and wavelength of the emitted light, to make the whole system more effective for marine animals. The whole pack of genes in wild *Vibrio fischeri* is stated on 9kb plasmid region and separated from regulative region (Meighen, 1993)

The firefly luminescence from (*Photinus pyralis*) is encoded by *luc* gene and it transports energy from ATP to D-luciferin so oxyluciferin, AMP, and CO_2 occur (Figure 4). The reaction is inducible by adding of luciferin or ATP and works only in

presence of Mg^{2+}. The light emission has very high quantum yield ~0.88. The light is induced at 560nm (Hakkila et al, 2002). The reaction occurs only in the presence of exogenous ATP that is why the reaction in not continuously produced but can be started just at time that is needed for scientific testing. In addition, the action is less stressful for cells due to not involving the whole light-emitting apparatus all the time.

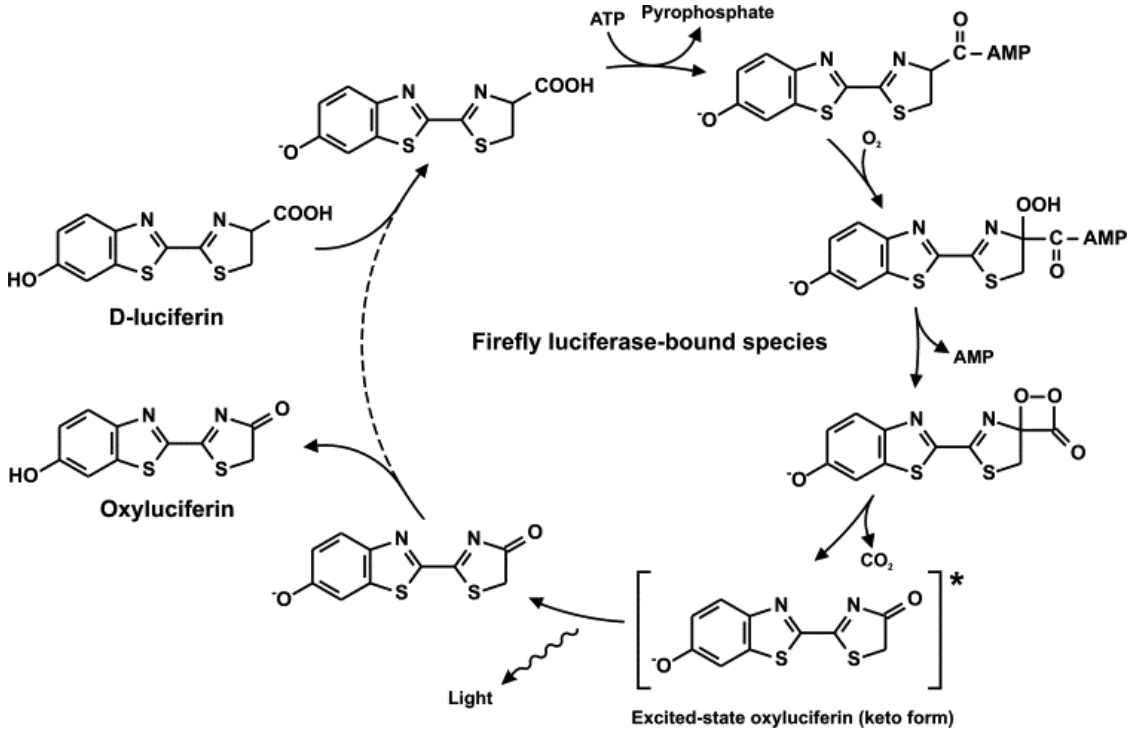

Figure 4. *The reactions cathalyzed by product of luc gene. The * symbol indicated the electon excited state. (Roda et al, 2009)*

One of the most important reasons why the luminescence system is so widely used, considering that it is occurred in the same time as the GFP, is its time independence. The visible and the detectable light occurs only at the moment of reaction and the effect is not cumulative. It means that there is a possibility to get the information directly at time it creates and measures the intensity at one particular the condition. Another important reason of using of the luminescence in experiments is easy light penetration through semitransparent substances and its safety for living tissue. Of course, the activity of light can barely be seen inside a rat, for instance, with bare eyes, but there is some quite sensitive equipment to investigate and measure the emitted photons. So there is no need to cut the body and make biochemical testings to check the position and the activity of, for example, a drug inside. It helps to decrease amount of lab animals. Safety for living tissue is regarding to nondestructive action of light in comparison to thermal, for instance, and also due to enzymatic safe – they are not harm to the native proteins of the body and then do not impair to the normal reactions.

2.2.0. Methods of detection

The convenient physical methods that are mentioned in the Dutch protocol, 2000 allow good quantification of total pollutant, but it does not cover process of bioavailability that is extremely important, for instance, for heavy metals.

Table 1 shows the detection limits of the classical methods for heavy metals described in this study.

Table 1. *Detection limits of physical detection methods. (based on Kohler et al, 2000 and WHO, 2011)*

Analyte	Test method	Detection range
Mercury	Atomic absorption	2.5-50 nM
Lead	Atomic absorption Inductively coupled plasma atomic emission spectrometry Atomic absorption graphite furnace X-ray fluorescence	10 – 50 nM 40 – 200 nM 5 – 50 nM 15 – 1.5 μM
Zinc	Atomic absorption, chelating Atomic absorption, extraction	76-30 μM 0.3-3 μM
Cadmium	Atomic absorption direct Chelation-extraction Differential pulse anode stripping voltammetry Atomic absorption graphite furnace	445 nM-0.2 μM 44.5 nM-2 μM 10 nM – 1 μM 18 – 90 nM
Nickel	Atomic absorption	12 nM

Atomic absorption spectroscopy is the most widely used technique for heavy metals detection and it is the one that was used in the study so it is described more precisely.

2.2.1. Atomic absorption spectroscopy

Atomic absorption spectroscopy or AAS is a measurement of absorption of radiation by free atoms. The sample should be pretransformed to gaseous state by various methods. The light for detection comes from ultraviolet and visible spectra.

Atomic spectroscopy technique usually includes atomic emission, atomic absorption, and atomic fluorescence spectroscopy. Atomic absorption spectroscopy is a measurement technique based on absorbance a portion of energy by an electron and so the atom comes from a ground state to an excited one. So, in some frequency, the intensity of the transmitted light drops. Tables of oscillator strength are available to allow a comparison of transition probabilities for a given line and a given element. Maximum value of the absorption coefficient on a given frequency is called K_{max} with a width of a line $K_{max}/2$. And helps to investigate the composition of complicated materials

Another rule allows correlating absorption and concentration. It is based on both Lambert's and Beer's laws (Figure 5). Lambert's law: "Light absorbed in a transparent

absorption cell is independent of incident light intensity. An equal fraction of the light is absorbed by each successive layer of absorbing medium". And Beer's law: "Absorption of light is likewise exponentially proportional to the number of absorbing species in the path of the light beam".

So the incident beam of monochromatic radiation I_0 falls on an absorption cell of length *l*. The transmittance is given by

$$T=e^{-klc} \quad (2).$$

So considering (2) comes

$$\log_{10}(1/T) = \log_{10}(I_o/I) = \alpha lc \quad (3)$$

and

$$\log_{10}(I_0/I) = A \quad (4)$$

where A- experimentally measured absorbance, so

$$A= \alpha lc \quad (5)$$

It means a linear relationship between absorbance and concentration.

Atomic absorption method requires a prior calculated calculation graph of the interested compound (αl as a slope for graph). So after getting the absorbance of the sample by the experiment, its result is just extrapolated to the concentration on the curve.

There are two main variants to vaporize the compounds: flame and electrochemical atomizers. Flame atomizer was created earlier and it is cheaper. But there are some problems in using refer to unstable temperature in different places of gas burner and also because of difference of flame sources. For instance, nitrous oxide–acetylene flame is hotter than air-acetylene. By the same token, flame on the top of the burner decomposes molecules less (to atoms) than on the bottom (to ions) as well. Another problem is a creation of side-products like oxygenation of samples with are, hence it needs modifications of burner with inert gaze cameras. (Welz et al, 2005)

Electrochemical atomizer is an electrically heated device such as graphite furnace or rod. This system is more stable and so the results are more reproducible. (van Loon, 1980)

On the current date here two types of monochromator source for AAS: line source (LS-AAS) and continuum source (CS-AAS). Line source is a situation when one radiation source emits spectrum that narrower that absorption lines. So several lamps are needed to cover the whole spectrum of UV-Vis light. Whilst in CS-AAS sources cover the spectrum that required for all elements. (Welz et al, 2005)

Limit of detection of this method is quite high and normally about several μg for heavy metals. For example, copper limit is about 1μg.

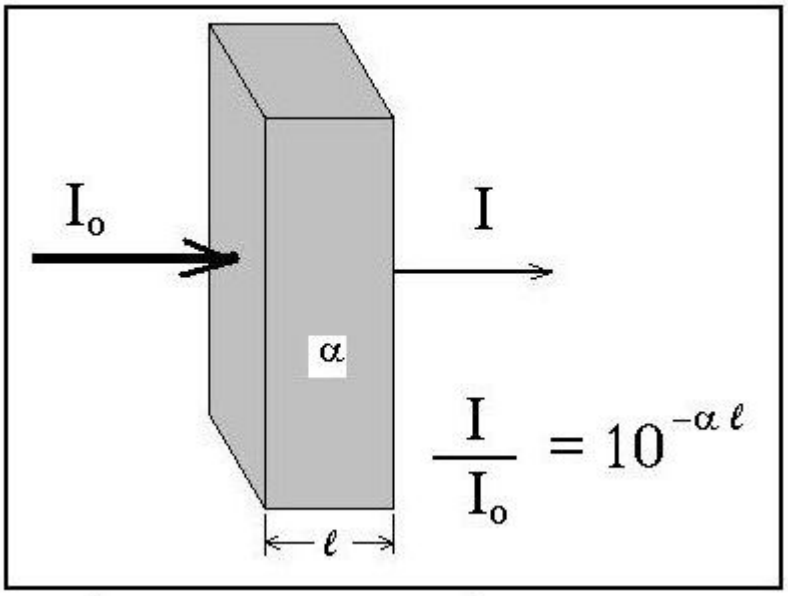

Figure 5. *Atomic absorption cell of length l with α as constant for given system and c is a concentration of the analyte. I_0 is an initial beam of monochromatic radiation and I is the rest of intensity of monochromatic beam. (Bengston, 2010)*

2.2.2.0. Biosensors

"A biosensor is a measurement device that composed of biological sensing part and transducer element that produces a measurement signal" (Daunert et al, 2000). In other terminology, "a biosensor is a coupling of a biological material with a microelectronic system or a device to enable a rapid, accurate, low-level detection of various substances in body fluids, water, and air" (Belkin, 2003). So there is a competitive point of view which does not accept the biosensor as a device but as a biological part of a device. There are three major types of sensing components: molecular, cellular and tissue.

2.2.2.1. Molecular

Molecular component is composed of specially designed biocatalytic proteins-enzymes or of bioligands to bind to the detecting compound – lectins, nucleic acids, sometimes antibodies. The transducers are needed to transform information to quantifiable signal and usually they are electrochemical, optical or thermal but the last generation involves piezoelectric or magnetic ones. Presence of mediators-intermediate compound that transports redox potential between transducer and the recognition element also interfere to the system behavior. (Struss et al, 2010)

With dependence of mediator and immobilization nature, molecular biosensors are divided into 3 generations. First generation is just a combination of a sensing element and a transducer, that are polarized to the proper value of potential so it can reduce the oxygen or oxidize OH^- group in the detection molecule. Second generation has an artificial freely diffusing in the sensor redox mediator. The mediator has just exact potential ability to regenerate the redox center in the molecule. The mediators can be one- or two-electron and usually has an inorganic part in the structure so they have good self-exchange rate constant. Third generation has a strict mediator-sensing element complex. So there is a direct electron transfer occurs between transducer and the complex. (Castillo et al, 2004)

For measurement of heavy metals there are some adapted proteins already existing. Some of proteins have a broad range of detection like urease which can detect Cu^{2+}, Hg^{2+}, Zn^{2+}, and Pb^{2+} or cholinesterase for Pb^{2+}, Cu^{2+}, and Cd^{2+} or L-lactate dehydrogenase. Or on the other hand, some specific responsible proteins can be used like merR for detection mercury as a product of the *merR* gene – the same that is used in this study. (Castillo et al, 2004)

Antibodies are another approach to detect pollutants. Their epitopes can be designed to any parts of molecules or to complexes of metals with bovine serine albumin or EDTA. There are different methods to check the amount of bounding molecules from plasmon resonance to ELISA protocol but all of them very sensitive and highly specific. (Verma and Singh, 2005)

In vitro cell-free biosensors can be applied in situations when there are some issues in cell machinery that block permeability through cell barriers or when something in the cell does not allow the sensing part work correctly.

2.2.2.2 Cellular

All methods that were described above are rather complicated and expensive, mostly because of need to refine the proteins. And, in addition, the highly purified proteins are unstable in room temperature. But the worst that they cannot really define the bioavailable concentration of pollutant – the concentration that can be nonharmful for living objects just due to partial penetration of chemical inside the cell. So the negative impact on system is rather overestimated with presence of "bare" sensors and data does not reflect the reality. (D'Souza, 2001) And the cellular biosensors are the way to remove the impact of this disadvantage.

Another important feature is that the molecular biosensors can express only the end point of the test and cannot be involved in long-time testing with flowing material. While the whole-cell sensors adapted for this situation, partly because they can grow and the luminescence system, in addition, allows obtaining data at any time point. Extra positive impact of the cell division is that the signal increase with every duplication.

Cellular or whole-cell biosensing methods are mostly based on presence of chimeric proteins combined from promoter region which reacts on presence of compound in the environment and also a reporter protein. The whole biosensors can be also divided to compound and effect-specific. In this study, all sensors are compound specific-so they react on particular elements and molecule, instead of the whole spectra of the environment. Another issue is that that the cell should be tolerable to toxic chemical or has genetic modified mechanisms to let it go through the cell wall and moves out. Usually, it are some kind of cellular pumps or binding protein involved. (Galuzzi and Karp, 2006)

Figure 6 is a scheme of an induced type whole-cell sensor. The analyte meets the appropriate receptor on the cell surface. Then it can pass through the membrane in a pump or with phagocytosis or just activate a system of second mediators. Nevertheless, the information about presence of the compound comes to the effector gene and activates its transcription and further synthesis of obtained mRNA. Reporter part of the proteins after folding creates a detectable signal.

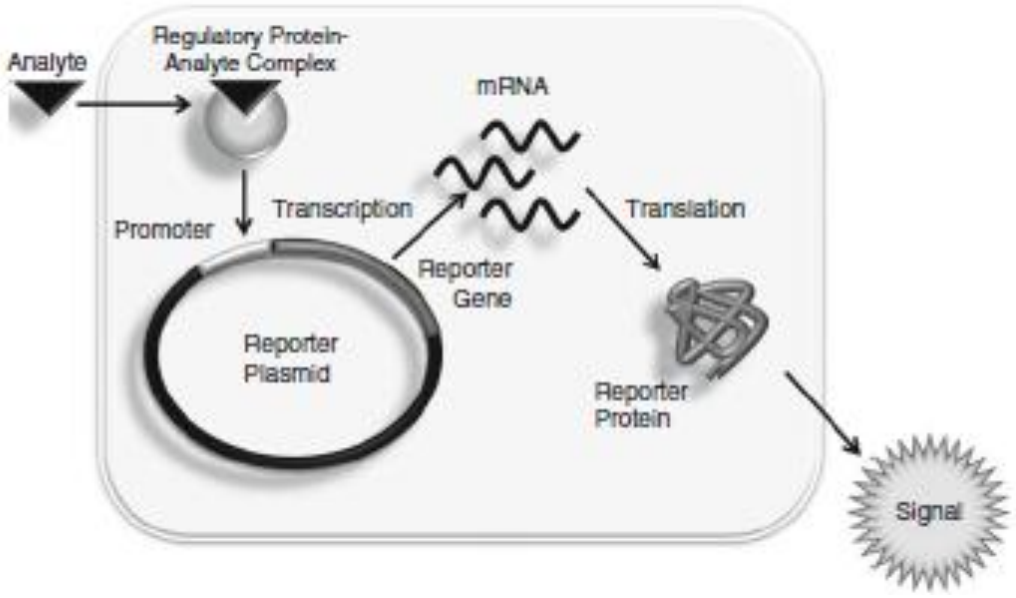

Figure 6. *Scheme of compound-induced (activator type) whole-cell biosensor. Struss et al, 2010*

One important system of detection is based on heat-shock proteins. These proteins are normally occurs in every cell in dangerous or undesirable conditions and their can cause cell death or simply decrease its metabolic rate to keep the cell as much intact as possible. The heat-shock proteins can work as chaperones, as proteases for protein denaturation, or as effector proteins that activate the protein synthesis. (Young et al, 2004)

The *rpoH* of *E.coli* is the most studied one. Its product σ^{32} works with more genes (~20) that react to promoter regions of other heat-shock proteins. So it has a regulon. It also starts activity of DNA-polymerase V (E) which serves for genetic mistakes removing. The feedback of the system goes with the end product of the σ^{32} – they involves in destruction of poorly folded proteins and so if there is no protein to cut, the signal returns to *rpoH* and the heat-shock proteins stop being produced (Missiakas and Raina, 1997).

Except the heat shock the cell can react to a variety of stress caused by starvation or different types of damages. Even the stationary phase of growth (*rpoS*) or lack of membrane (*fadR*) regulates the further cell behavior with this protein type. (Daunert et al, 2000)

On the other hand, there is also a system based on human liver cells HepG2 with CAT reporter system. The variety of stress response causes is quite high – toxic and nontoxic ones are xenobiotic, DNA damage, antioxidant response, heat-shock, protein damage, and heavy metal (MT 11A stress gene) (Todd et al, 1995).

Unspecific detection system like Microtox® based on ability of non-transformed organisms (*Vibrio fischeri* in case of Microtox®) to report their exposion to the chemicals. (Abbondanzi et al, 2003). The Microtox® was in use in waste water treatment plant but sometimes there were situations when there were too much hazardous agents in

the sample so the cells died. As a result, the sensing device was improved by connection with another vessel with the same cells. Now in the two minireactor system there is one measurement vessel where the testing samples are pumped in and where the bioluminescence is counting. In the second vessel, new cells are growing and then they continuously are flowing to the first reactor, if necessary. This system can report any failure in water treatment on early stage.

2.2.2.3 Biosensing elements and chimeric proteins

The bioluminescence reporters are covered in a separate chapter. But there are also other ways to get a feedback on the system with other reporter proteins. The promoters and the response elements are taken from naturally adapted organisms – ones who are able to grow in the conditions of high contamination. They can be performed as changing color (β-galactosidase) or emitting light systems (bioluminescence or fluorescence).

β-galactosidase encoded by *lacZ* gene and catalyzes the hydrolysis of β-galactosides. It has very fast turnover and can be detect by colorimetry, histochemistry, electrochemically or via luminescent and luminescent methods. The idea that lied in the basis is changing of substrates. But the reporter protein has low sensitivity and narrow dynamic range – the cells need to grow and form colonies. (Daunert et al., 2000; Kohler et al, 2000)

Green fluorescent protein is found in a jellyfish *Aequorea victoria*. It is a short Ca^{2+} binding protein that can be folded even in prokaryotic systems to the highly stable "barrel" formation. Because the protein is cumulative, it can be used for detection of even low amounts of the gene product, but it also means that the response is not precise and increase with time. GFP protein is the most usable reporter system on a current moment and it also has different modifications with different colors so it is possible to track several proteins in one cell. Quantum yield is very high also – about ~0.88. (Struss et al., 2010)

There are two variants to perform chimeric protein to work - activating and repressing ones. In activating mechanism, regulating protein is present all the time but it starts to work only after appearing of the compound (Fig. 7). The compound can change the protein conformation or, for instance, couple it, etc.

The second type is a repressing one when inducer (e.g. pollutant) binds to repressor and removes it from path of RNA-transferase so the whole protein is synthetized (Fig. 8) and so after proper folding the whole system response. This way is mostly used for evaluation of the total toxicity or the common factors.

The activator type is more adapted to detect low concentrations of toxins – production of light with there is at least anything in the media, while the repressor type may be more valuable in high concentration test when the light stop to be producing at the critic point and there is possible to mark the threshold concentration.

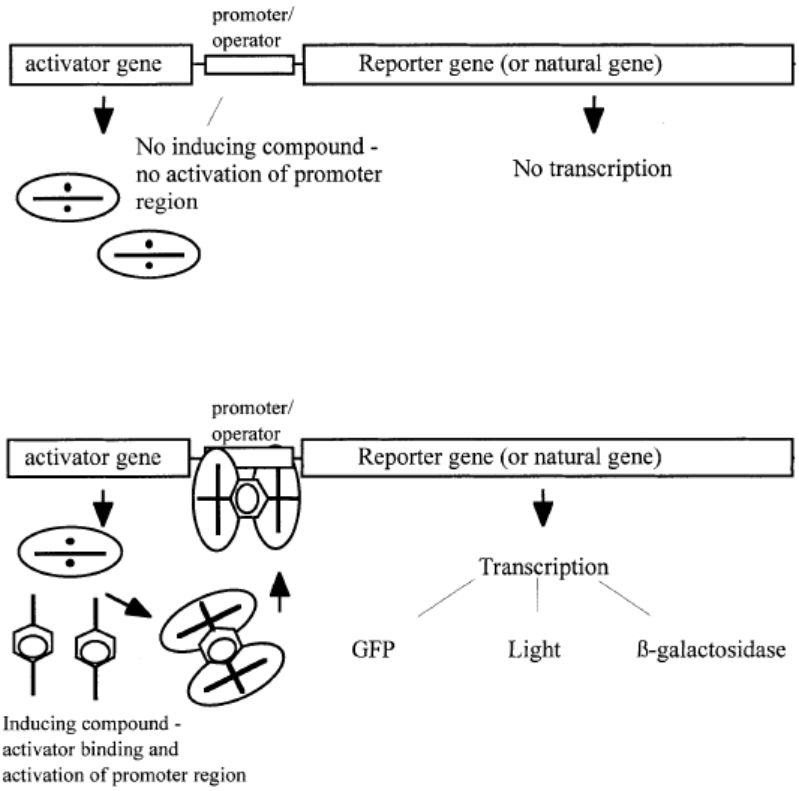

Figure 7. *Scheme of activator type of the reporter gene. (Hansen and Sorensen, 2001)*

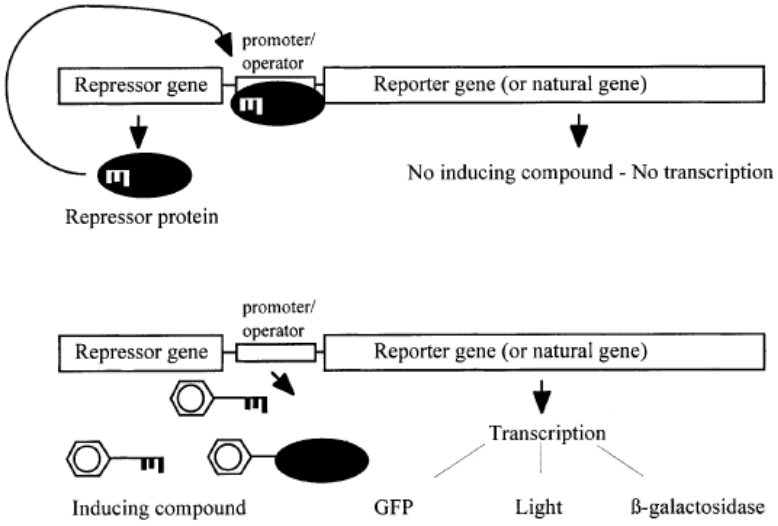

Figure 8. *Scheme of repressor type of the reporter gene. (Hansen and Sorensen, 2001)*

As already said, regulation protein and promoters as well are taken from naturally species. Usually, it codes pumps that transport ions inside a cell or a promoter of degradation pathways in case of organic pollutants. The most common situation for metals is binding to thiol or methyl-thiol groups of protein (Verma and Singh, 2005), which allows passing through the cell barrier and then release.

There are also several factors that affect on the cell reaction. It is embediment of the biosensors, or the composition of the medium where they grew, or the method how the cells are stored in time. The easiest way is a prolonged storage of the cells at -80 and using as a regular cell culture, with sequenced growth. This method requires quite a lot of time, because cells need to inoculate every time of using, and also it occurs that some cells are sensitive to glycerol compound which is widely used as antifreeze agent. Another method is more promising – the lyophilization or freeze-drying when the cells are deeply freezed with the following sublimation of water in a vacuum dryer. The cells are remaining intact at room temperature for a week and at -40 and lower for several years. The cells can be revived simply with adding of water and incubation at room temperature for several hours. If the cells were extra stabilized in lactose, the cells can easily grow up in shaker to increase their concentration. The last, method is using naturally occurred preservatives, for example, spores formation of bacteria *Clostridium spp.* (Galuzzi and Karp, 2006).

The immobilization can be made in natural, like gelatin or albumin, or synthetic polymers, like polyacrylamide, different resins and hydrogels. The cells can be immobilized there with different techniques: entrapment, covalent binding, crosslinking (or combination of the two previous ones), photo cross-linking, freezing and thawing, or γ-irradiation. So the cells can be either trapped into the system and their surfaces remain intact, or on the opposite, there can be firm bonds between the cell and the material. This is a reason why the synthetic polymers are so handful in the work – their chemical chains can be designed in any way and has as much sites of attachment as needed (Uhlich et al., 1996).

The one of the most important limitation on the immobilization techniques is a creation of an additional barrier for the ions penetrated inside the cells. This disadvantage can be minimized with the open pore entrapment method, when the testing sample has a possibility of a direct contact to the cell (D'Souza, 2001).

Disadvantage of the whole-cell sensors, in comparison to molecular ones, is their slowness. It is because the molecule should pass through the cellular membrane first. So the cell permeability should be increased with divergention agents or with placing the sensing systems into the periplasmic space. (D'Souza, 2001, Rani et al., 2008). In the same position, this phenomenon is also an advantage – such mechanism protects the intracellular apparatus and enzymes in complicated conditions and allows them even grow in the high toxic environment.

The reactivity and stability depends on a host strain as well. Some cell walls transmit more ions than the other, some hosts are more stable in different conditions, or requires special combination of temperature, pH and a moon phase to start the reaction.

Another problem is the less specificity of the cells. One protein or one pump can react with different compounds so the signal interferes in presence of other chemicals. (D'Souza, 2001). For instance, the sensor that designed on *cad* operon basis also indicates zinc, lead, and nickel in the environment. So the response is actually a sum effect of all of these. Also, at last, the other some factors are effect on activity of the enzymes, such as pH, oxygen supply, temperature. So the system is hard to be unified and the whole idea should be adapted for high-throughput and a protocol should be created (Virolainen, 2012).

Arsenic reporter system is one of the most studied and widely used in laboratory practice. *Ars* operon codes efflux pump that can remove arsenite and atimonite from a cell. The ArsA protein is an ATPase that reacts on a presence of the chemicals and provides the energy for the transporting of ions through the membrane via the ArsB pump. The ArcC protein is a helper that reduces As(V) to a less toxic As(III) form. The last protein is the ArsR which is a suppressive regulator. It binds to the promoter region of the *ars* genes and stops the expression. (Roberto et al, 2001)

There are 2 variants of the cell sensors were used in this study: cadmium sensing system based on *czc* operon and mercury sensing on *mer* operon.

Cadmium, unlike of the As and Hg, can operate with the already existed pumps of Mg or Ca to get into cells. But the mechanism of protection resembles the arsenic one – the efflux pumps remove the ions out of the intracellular space without its reduction. There are several genetical mechanisms in bacteria to achieve it – from *Staphylococcus aureus* (*cad* operon), from cyanobacteria (*sml* operon) and from *Gluconobacter* bacteria group *Rasltonia eutrophus* (*czc* operon). These operons are adapted to Pb, Zn, Ni, and Co. The cadmium operon *cad* codes two proteins: CadA, which is a pump for removing ions out of a cell, and CadC, which is a P-type ATPase that regulates CadA. (Daunert et al, 2000).

There is also a *czc* operon and *pbr* operon, which form with efflux antiport system with Ca^{2+} and as whole resemble the *cad* operon in action but involve CBA transporters. The completely different method is performed in cyanobacteria, with metallothioneines that bind ions prior extrusion. This method resembles the one in eukaryotes – only there is glutathiones instead of metallothioneines. (Diel et al, 1995)

Hynninen and colleagues (2010) from Turku offered two types of sensors based on *cad* and *czc* operons and *lux* reporters where pump genes from the promoter system had some mutations so they did not work properly. It was done with a hypothesis that the efflux pump decrease amount of intracellular ions and so decrease the signal. The hosts were chosen to be *Pseudomonas putida* because of its high stability in environment systems. The test was performed on Zn, Ni, Pb and Cd standard curves and as field test

the Zn contaminated soils were chosen. The cells showed good results with very low detection limits and so considered as successful.

mer operon was used in several studies, such as Selifonova et al, 1993; Virta et al, 1995; Lyngberg et al, 1999. It is again an efflux pump with facilitate proteins, just like the cadmium or the arsenic sensor. *Mer* operon of gram-negative bacteria usually composed of *merR* gene which codess a regulatory protein, products of the *merP* and *merT* are transport protein which isolate and transport the ions inside a cell to reducing enzymes. They work in a periplasm and inside a cell respectively. Their activity occurs with cysteine residues. *merA* gene and its product MerA protein are relates to mercuric reductase that change ionic mercury to its elemental form. The reaction is HADPH-dependent and so creates additional stress on the cell because the energy can be needed in other cellular activity. Some bacteria also contain a *merC* gene which codes a membrane assistance protein that facilitates the penetration through the cell wall. But if there is methyl mercury, for example, in the system, there is an extra gene *merB* needed which is an organolyase and can cut out the organic parts from the molecule. In absence of mercury in the cell, the *merR* protein binds the P/O region and prevents the synthesis of other genes so the system is repressor regulated.

Because mercury is extremely dangerous and can come to human organism from many ways there is a great majority of articles that represent methods of its detection. The whole spectra of reporter proteins were used. According to the Hakkila and colleagues (2002), the *lux* reporter protein provides the best results for both IF and the range of worked concentration. The *luc* construct appears to be good as well but it does not work at high amounts of ions. Response of GFP-contained plasmid, on the opposite, performs in a wide range of concentrations but the activity is lower in several times. In this study, it was used the sensor obtained from Rantala et al (2011) study which is the same as Ivask et al (2002). This plasmid has both the *merR* and *merB* genes and the *lux* gene reporter complex. The figure 9 represents the *merRlux* construct without the organolyase gene.

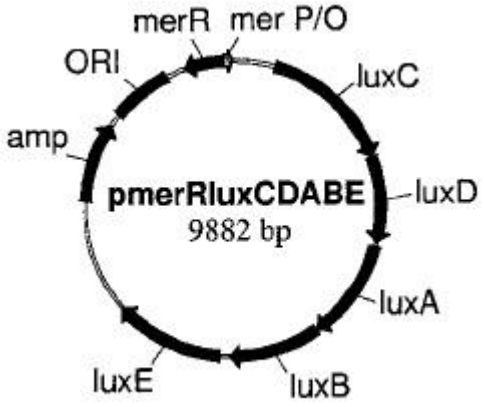

Figure 9. *Scheme of pmerRlux plasmid (Hakkila et al, 2002)*

There are a lot of variations of the constructs for sensing mercury and bivalent metals were designed.

There is also the plasmid created by Nagata and colleagues (2010) with their pHYmer-lux plasmid. The difference is that it reacts faster – about 30 minutes are needed for reaction.

A group of Chinese researches, Wei et al (2010) developed a chromosomally based sensor. There is another technology preformed – it contains *merR* and plasmid pUT-ME later transformed to chromosome element. The minimal detection limit is 200nM and it also works for bioavailable mercury.

Shetty et al (2003) also create a lead, zinc and cadmium sensor, but the methodics is quite complicated: they expected to use cell lysis. The researchers combined part of *znt* operon with *rs-gfp*. On the other hand, there obtained a detection limit around 10 pM/l, which is extremely low.

There is also a smt operon for primarily zinc detection, but it can also be applied to copper and cadmium assays. Erbe et al, 1996 for instance combined the *smtB* with *luxCDABE*. But the detection limits were not good in comparison to the similar studies.

2.3.0 Heavy metals

'Heavy metals' is a name for a group of metals and metalloids that have been associated with contamination and potential toxicity and ecotoxicity. Such metals are have density larger than 4 g/cm^3, relatively high atomic weight (in comparison to sodium), and, as the most important, at some dosage they interfere with metabolic pathways and break them through wrong folding of proteins or blocking of enzyme activity. Duffus , 2002 shows that term "heavy" does not have any chemical basis and provides it as an obsolete. And so further using of this word can cause misunderstanding and lead to problems in investigation of the toxicity mechanisms.

Dr. Alina Kabata-Pendias in her studies divided all the heavy metals according to their activity in biotest. If heavy metals inhibit cell activity in concentrations less than 1 mg/l they are assumed as high-toxic - Ag, Be, Hg, Sn, Co, Ni, Pb, Cr. If the metals inhibits a biotest samples in concentrations 1-100 mg/l they are so called semi-toxic - As, Se, Al, Cd, Cr, Fe, Mv, Zn. The last group – Ca, Mg, Sr, Li – inhibits biotest in concentrations more than 1800 mg/l and is low-toxic. (Kabata-Pendias, 1991)

In most cases, the heavy metals contact with proteins through some reactive groups and change their configuration and so simply break them or do not allow meeting their functions properly. Another way of harness is cancerogenic when the agent blocks or damage pathway of programmed cell death, so the mutated cells do not die with normal immune responses but turn into tumor.

Nevertheless, some heavy metals, so called trace elements, are necessary for living beings including animals and plants. Such elements like Zn and Cu have to be included into ration to proper function of enzymes. Zn will be discussed further but, for instance, Cu is shown in cytochrome *c* oxidase and in hemoglobin-like protein in mollusks (Greenwood and Earnshaw, 1997). In these part heavy metals are used as Lewis acids that can be used in hydroxylation. If the proteins are necessary for a body it is usually transported by albumin in a blood.

Another problem for the detection and the evaluation of the toxicity is that some chemical compounds are dangerous or carcinogenic only in form of a salt or, on the opposite, in form of a metal. For instance, chromium is used and considered to be safety in stomatology, while chromate is carcinogenic compound (Duffus, 2002). But usually the organic compounds of the metals are the most dangerous because they have the liposoluble part so they can penetrate barriers inside a body and through cell walls.

The last problem is that heavy metals change their activity in combinations. For example, addition of zinc or cadmium into environment with high amount of copper, increase toxical effect of the Cu. This phenomenon is shown for plants and soil bacteria but have not investigated in humans yet. (Kopittke et al, 2011)

Heavy metals are a part of various products of daily usage. Such the mercury is still used in thermometers and barometers because of its significant physical properties. Or another way of leaking of the chemicals to the atmosphere is a side contamination in

products where the metal is traced. For example, the lead that used in form of tetraethyl lead for reaching necessary octane number of gasoline. So the end product has some traces of the elements that are released when the oil burn in an engine. Some contamination also appears during the mining and cleaning of the oar material. And the last way of exposing into environment is a natural erosion of reservoirs.

This chapter covers heavy metals that are studied in the research: mercury (Hg), cadmium (Cd), lead (Pb), zinc (Zn), and nickel (Ni). Table 2 shows normally occured health problems and sources and sites of contamination.

Table 2. *Sourses of heavy metals. (Rami et al, 2008)*

Metal	Sources	Disease
Lead	Mining, coal, automobile, paper dyeing, petrochemicals	Learning disability, mental retardation
Chromium	Leather/ tanner, thermal power plant, mining fertilizers, textile photography	Bronchial asthma, allergies
Cadmium	coal, nuclear and coal power plant, batteries , ceramics, toys	Itai Itai disease
Nickel	Mining, coal, power plant, phosphate fertilizers, chocolate, automobile electroplating	Dermatitis, Pneumonia
Mercury	Mining, paper and pulp, coal power plant, cement, electrical equipments, pesticides cosmetics	Minimata disease
Zinc	Phosphate fertilizers, distillery, pharmaceuticals	Fever

2.3.1 Mercury

Mercury is a metal with atomic number 80. Mercury is stable in ^{199}Hg, ^{200}Hg, and ^{202}Hg isotopic forms. It is liquid in normal conditions and most likely works in 1 and 2 oxidation state but also there a 4 form can be found. Mercury is heavier that water and considers to be a dielectric. Human MPR dose of mercury in total is 0.9 μg/kgbw/day (Dutch protocol, 2000).

In nature, mercury is shown in a metal form or as a part of allows with gold and many other metals. But from the chemical point of view mercury does not tends to react with acids but very strong acid oxidators dissolve the metal with creation of sulfuric, nitrate and chloride salts. (Greenwood and Earnshow, 1997)

The metal forms are not so dangerous for organisms after swallowing – it is almost not absorbed by gastrointestinal tract, while the traces and vapor forms of mercury

(HgO) that produced from the metal exposed to air oxygen can harm a lot. The contamination appears after reaching 0.25 mg/m^3 in the environment and can leak to a body with breathing and microdamaging of skin. The poisoning of the vapors caused asthma and pneumonia like symptoms in combination with the damaging of gastrointestinal system. Without an appropriate medical treatment the exposion can cause death.

In the body mercury mostly can react with $–NH_2$, $-CONH_2$, -SH, -COOH,$-PO_4$ and to the Zn and Se compounds (Melnick et al, 2010) and so causes breaking of the 2^{nd} protein structure or the dimerization of proteins, which leads to a misfunction of enzymes and a breaking of the cell metabolism and cell death. The ions that have not been involved into the metabolism accumulates usually in liver, kidneys or brain (for organic compounds). (Zafir et al, 2005)

Organic compounds of mercury element can be shown in methyl, ethyl and dimethyl. All these compounds are liposoluble and can pass through skin and reach intracellular compartments. The main problem is that they can also pass through blood-to-brain barrier and accumulate in brain tissue and so cause psychiatric problems. The dimethyl compound is the most dangerous but happily it is presented only in laboratories. Ethyl and methyl can be found in nature and the ethyl is estimated to be more harmful than methyl one. (Rooney, 2007)

Inside the organism mercury can bind to the diffusible thiols which are highly transportable across membranes. And also can cause a molecular mimicry when the complexes of the element with proteins have homologous to some other natural complexes. Because of this it can use cellular machinery for transportation (homocysteine conjugates with methylmercury are substrate for transporting in hOAT1 transporters). (Rooney, 2007)

Wood (in Boening, 2004) shows 6 ways of bacteria to interfere with mercury:

1. Efflux pumps that remove the ion from the cell.
2. Enzymatic reduction of the metal to the less toxic elemental form.
3. Chelation by enzymatic polymers (i.e., metallothionein).
4. Binding mercury to cell surfaces.
5. Precipitation of insoluble inorganic complexes (usually sulfides and oxides), at the cell surface.
6. Biomethylation with subsequent transport through the cell membrane by diffusion.

The last one is the most dangerous for the environment because this mechanism renders the mercury more toxic to the organisms with higher organization, including mammals.

In dependence of the velocity of the intoxication, for human there are two different sets of symptoms. Soon after rapid poisoning there a fever, a severe headache, an asthenia, and nausea occur. During gradual and prolonged intoxication the symptoms mostly refers to psychic dysfunctions like an apatia and an emotional instability and

also somatic problems as well – a malfunction of the cordial rhythm and a hyperfunction of the thyroid gland. But the most crucial influence the mercury exert on development embryos, where it leads to a severe retarding or to problems in bearing. (Goetz, 2003)

A treatment of a mercury intoxication is made with thiols groups as well. The DMPS and the DMSA use mercapto groups for an attachment and removing of ions. But these chemicals do not pass barriers inside a body and there is a reason to use alpha-lipoic acid. Zn, Se and fiber intake can be an extra help in removing Hg, especially from gastrointestinal tract. (Rooney, 2007)

The main sites of contamination of mercury are the industrial minings, the chemical discharge, the electricity production, and the contamination from products like thermometer. Mercury can be accumulated in marine animals and fish and enter human body with their consumption. Therefore the populations with high percentage of marine products in dietary have the highest exposure of Hg. Chinese population, unfortunately, are in danger as well now – about 12% of the current air emission of mercury is made in Guizhou in South-Eastern China (Zahir et al, 2005).

2.3.2 Lead

Lead is a chemical compound that takes the 82^{nd} atomic number and it is a post-transition metal and refers to D group of elements. Lead has three stable isotopes ^{206}Pb, ^{207}Pb and ^{208}Pb. In nature it is found as part of ore and appears as silver metal. In chemical reactions lead can usually lose 2 electrons so become Pb^{2+} and can also create 2 or 4 coordinative bonds. There is also a Pb^{4+} oxidative state but it can occur only in highly acidic solution (Greenwood and Earnshow, 1997). Maximum permit limit of lead intake is more than mercury – 3.6 μg/kgbw/day (Dutch protocol, 2000).

Lead is a necessary compound in our life which is used in gasoline production, building construction, batteries etc. Normal people confront to lead every day and amount of lead buried in the landfills is huge.

Lead is dangerous in any chemical composition, because ions are the reactive agents of the compound. Another problem that the direct effects of lead have been not described. So the treatment and reacts are symptomatic and based on chelating of the metal. But just as mercury it has even more aggressive organic variant. After ingress of the ions in body it appears that it starts to behave like classical chelating agents - it accumulates smaller molecules around or bends proteins. The reaction are possibly based on $–CH_3$, -OH, -SH, and $–NH_2$ radicals. (Flanagan et al, 2008)

Available lead is stored in soft tissues and can release rapidly, but in bones and corneous tissues like hair or nails the chemical is bound much tighter and does not affect the health so much. Nevertheless, bone-bound lead can release in long time after and cause chronic effects.

Lead shows a broad range of effects including a genotoxicity and a blocking of a heme synthesis. Another variant is a blocking of a glutamine neurotransmitter activity that involves in signal moving so the nervous system does not work properly. All these reactions can lead to renal or liver, and sometimes polyorganic, failure. In any case, the excess of the compound accumulates in tissues. (Flanagan et al, 2008)

Symptoms depend on the variant of the toxical activity, the duration, and the dose and also vary from patient to patient. The basic symptoms are a headache, an abdominal pain, a memory loss, and a kidney failure. Additionally, there can a weakness, a losing of memory, seizures, and a coma occurs. For children, lead explosion usually leads to mental disorders and an arrest in development. (Rossi E., 2008)

One of the reasons why lead is so hazardous is its high availability in nature and wide using in manufacturing, including lead paints. So people are subjected to the chemical contamination more often than to mercury, for instance. (Needleman, 2004)

The most danger in lead is caused by its incompletely recycling so the traces are left in environment and also by using lead as an agent for increasing quality of gasoline so after burning in an engine the lead residuals left in air. The industrial urban territories are endangered most of all.

The best variant for the ecological evaluation of bioavailable lead in sites is monitoring of amphibian populations through the time. Because these animals are exposed lead both in water and on earth during their cycle of development, their populations are very sensitive to all heavy metals, including lead (Arricta et al, 2004).

2.3.3 Nickel

Nickel is chemical compound with atomic number 28. It has three most widespread stable isotopes ^{28}Ni, ^{30}Ni, and ^{32}Ni. Nickel is a hard durable metal that refers to transition ones in the chemical table. It is found as silvery with golden tinge and very stable at room temperature. Nickel alloys are corrosion resisted and that is a reason why it is used as a part of stainless steels composition. Another important feature is a ferromagnetic activity of compound. In alloys with titan it forms Titanol® which tends to return to its form after bending and able to work at 37°C. And so it is used in surgeries and orthodontic therapies. In daily usage it can also be found in 1 and 2 euro coins and in tobacco products. (Greenwood and Earnshow, 1997)

Nickel does not naturally use in protein production in human body, but it can be found in plants, bacteria and fungi. There it is a part of enzymes such as useares, hydrogenases etc. No enzymes or cofactors do not use nickel in higher organisms. Nevertheless, decreasing of nickel in ratio during the development can lead to reduce of a growth, mental disorders and alterations in behavior in rats. Another important role of Ni is that is a cotransporter of iron in a gut and so that deficient of nickel decrease hemoglobin and cause non-cellular anemia. (Greenwood and Earnshow, 1997)

Nickel can chelate proteins because of the presence of coordinating bonds. But the main problem of occurring of the chemical in environment is its activation of immune

system. Direct contact between skin and nickel leads to a penetration of ions through the skin where the Langergance cells react on it, attract the T-lymphocytes and activate the antibody production. The antibodies aggregate on the Ni ions and start an inflammation process. This allergy type further can spread to lungs and cause synovial problems. The nickel-indused allergy can be diagnosed with localization of the irritation on the places with direct contact to nickel surfaces, such as accessories or watches. (Janeway et al, 2001)

Another important problem that Ni can replace Zn, Mn and Mg ions in activation centers of enzymes because they are competitive transporters. At last, nickel has carcinogenic activity as well.

Symptoms of nickel contamination can be divided in two parts – immediate and delayed. The immediate ones are a headache, a vomiting, an insomnia and a vertigo. Further it develops to a chest pain and later to a pneumonia-like hemorrhage. The last effect is a fibrinous intralveolar exudates that can cause a polyorganic failure and also a cerebral hemorrhage. (Ilic et al, 2007)

2.3.4 Cadmium

Cadmium is a transition metal that takes the 48^{th} atomic number and shown in 4 more or less stable isotopic positions of ^{110}Cd, ^{111}Cd, ^{112}Cd, ^{114}Cd. Cadmium has got properties that remind ones of mercury and zinc. Cd can be found in rechargeable battery devices in combination with Ni and takes part of a negative in the electrical cell structure. Electroplating is another part of usage because just like Zn, Cd has got very strong corrosive-resistance properties. Cd is also widely used in corrosive resistance paints in forms of CdS. (Greenwood and Earnshow, 1997)

Cadmium has got no relevant biological role in animals. There is some evidence of application of cadmium in marine algae. So due to this situation, the element is highly foreign to organisms. And even trace amounts of cadmium in environment can lead to chronic diseases.

The low concentrations of cadmium with a chronic exposure cause a removing of Ca^{2+} ions out of bones and so make them softer. Also a kidney and a liver failure come very soon after affection of high doses. Inhalation of cadmium, e.g. with cigarette smoking, damages the respiratory tract. Acute poisoning shows flu-like symptoms – dizziness, a fever, a cough and other respiratory problems. Nickel is a highly carcinogenic compound (Flanagan et al, 2008)

2.3.5 Zinc

Zinc is a post-transition metal that atomic number is 20 and shown in nature mostly with ^{64}Zn, ^{66}Zn, and ^{68}Zn. It is the 4^{th} metal in worldwide usage. Nowadays, Zn is mostly used in production of batteries (because of low standard electode potential) and

as anti-corrosion agent. Protective properties can be achieved with coating or as a part of alloy composition. The most well-known alloys are brass and bronze that both are a combination of copper and zinc but differs with ratio – brass has 60-70% of Cu while bronze reach 90% of Cu content. These materials are very ductile in comparison to copper yet save its electro conduction. (Greenwood and Earnshow, 1997)

Unlike of metal that described above, Zn is necessary for human beings in relatively high amount of daily intake – 8-11 mg/day. And around 10% of proteins of animal organism are able to conduct Zn in the structure, like the Zn-finger that can recognize DNA patterns and bend or cut the DNA strings in certain places and has a Zn ion in the core. So its deficiency leads to the DNA damage.

Zn is also used in the Zn-signaling pathways (Hajnal, 2003, Kaloyianni et al, 2006). In all these cases Zn coordinate protein molecules around and create flexible bonds.

If amount of Zn in food is not enough, at first it causes a losing of appetite because in normal conditions it binds to the leptin peptide producing cells and increases its amount. And it also can cause a diarrhea. This is a reason why the renormalization of Zn level is one of the first steps in the anorexia and bulimia treatment. Another symptom is a losing of the smell sense and also the eyesight, the taste, a depressing of immune system, memory and cognitive skills. Skin problems like lesions and acne and presence of spots of nails are the visual symptoms of the Zn deficiency.

Lack of Zn in men diet sometimes leads to crucial defects in sperm production and in the normal activity of a prostate gland. Synthesis of some anabolic hormones like testosterone, insulin and a hormone of growth depends on a presence of Zn as well. Daily intake can be achieved with dietary supplements in form of polyvitamins or monodrugs and with fortified food. (Maret and Sandstead, 2006)

Zn is dangerous mostly in forms of chlorides and sulfates. The poisoning of Zn, in addition to its chelating properties, is based on concurrent binding to the iron or copper transporters so the poisoning is tightly connected to the deficiency of these elements. So it leads to muscle slowness or rigidness and also to a non-cellular anemia. Because Zn regulates content of water in body, high amounts of the metal in an organism cause a strong thirsty and sometimes leads to a renal failure. Symptoms of the Zn poisoning are also ache in a chest, cough and dizziness. (Flanagan et al, 2008)

2.4 Slurry

2.4.1 Characteristic of slurry as material

Slurry, in general, is a suspension of solid particles in a liquid, as a mixture of cement, clay, coal dust, manure, meat, etc. with water (Collins English Dictionary). In particular case of bioslurries there is a high amount of bacteria and a high percentage of dissolved organic compounds such as proteins and carbohydrates and its condition is a disperse colloid solution of solid particles in organoacids. These particles can be composed with different metals and organic fractures. So the dispersed particles can condensate ions inside and create extra surface for the contact of the solid and liquid phases in the solution. As a result, the surface of reaction to microorganisms increases, and so a microbial activity is very large in such systems. It increases the leaching and velocity of the decomposition of organic matter. Thereby, the liquid in the slurry is rich with nutrients and microelements.

Except an increasing of the effective surface, presence of such small particles gives high viscosity properties to the slurry. So the solution is not even and homogenous, but there are some local increments of chemicals concentration such as enzymes or metabolites, which allow an effective consumption and a formation of colonial organisms. Also the viscosity causes also a slow penetration of gases which additionally increase a stability of parts.

Marcato and colleagues in 2008 have tried to estimate the distribution of the particles in samples taken from anaerobic digested conditions. They compared raw and digested slurry and found that the percentage of the particles with bigger diameter is larger in the digested slurry. It occurs because the small particles (1-60 μm) degrade first. Also they have tried to check the correlation between the size of the particles and the ionic composition of it and they found that Cu and Zn are trapped mostly in 3-25 μm particles and so amount of dissolved metals is higher in the digested samples (Marcato et al, 2008).

The main properties of the slurry composition can be found in such articles that show simulated animal waste solutions like Brown and Shackelford in 2007 have been used. The simulation does not represent the particle presence but they are based on real solutions of the waste water treatments plants. Table 3 compares the waste water solution with a deionized water. As you see the waste solution is reach with ions and the electrical conductivity (EC) is higher as well (Brown and Shackelford, 2007).

Table 3. *Example of compositions and properties of deionized water and simulated animal waste solution used in the article (Brown and Shackelford, 2007).*

Parameter	Permeant Liquid	
	Deionized Water	Simulated Animal Waste Solution
Electrical conductivity, EC (mS/m) at 25°C	0.20	606
pH	5.60	6.10
Dissolved oxygen, DO (mg/L)	7.80	7.80
Redox potential, Eh (mV)	191	51.5
NO_3^--N (mg/L)	0	0
NH_4^+-N (mg/L)	0	379
Cl^- (mg/L)	0	1697
Ca^{2+} (mg/L)	0	139
Na^+ (mg/L)	0	502
PO_4^{3-} (mg/L)	0	150
Ionic strength, I (M)	~ 0	0.059

Using of slurry can solve several problems in one time – it is not just a removing of huge amount of wastes but also an addition of fertilizing agents in soils or, on the other hand, a substrate for methane or other organic compounds production. And so for successful utilization of the slurry, it should be pretest first for determine the contaminating agents of both organic and inorganic origin.

Because testing of the contaminating agents is relatively complicated and requires some expensive technologies, there is an idea to attach the measurement of metal concentration to some easy to determine characteristics like pH, EC, redox potential, specific density, total solids, sedimentable solids, biological oxygen demand, chemical oxygen demand, total nitrogen (TKN), ammonium nitrogen (AN), organic nitrogen, or total contents of phosphorus, potassium, calcium and magnesium. Usually the evaluation works in combination of these methods and Moral et al, 2005 obtained data that EC has the better correlation to TKN or AN and so it can be used for crude but fast evaluation of them (Moral et al, 2005).

In some cases, heavy metals and other pollutants can be overestimated because not all the contaminant reaches an organism and harm the organism, but the whole amount of chemical is measured during physic-chemical mechanisms of testing like standard AAS or HPLC or new applied near-infrared spectroscopy (Ye et al, 2005). So there are several other way to determine whether the slurry is harmful or not. For instance, biological methods that were explained before, but in relation to the slurry it can be Daphnia magna (de la Torre et al, 2000). Or the biosensors that are showed in another chapter.

The concentration of heavy metals in manure depends on pureness of feeding stocks. Organic farm shows less contaminated ions in serum of cattle (Tomza-Marciniak et al, 2011) and so less contaminants in feces.

2.4.2. Application of slurry

Slurry can be used for fertilizing soil or for bioremediation of contaminated area or for production of some important compounds. The fertilizing occurs in different methods – first, the more prevalent way: the digested slurry and some soil are fermented together and then this mixture is added to the fertilizing area – composting. The second way is injection of the liquid fraction of the slurry into the soil layers (Chen, 2002). The main reason of slurry application is the extreme amount of bioavaliable ammonium and nitrates in it (Diez et al, 2001). Consequently, it decreases amount of inorganic fertilizers required. Another advantage of the slurry as a fertilizer is that it is needed in very small amounts and it helps to reduce soil loss (Gilley and Risse, 2000).

Another reason is that there are a great majority other salts and organic compound which create or maintain buffer conditions in the soil. The addition of soils and coexposition helps remove and assimilate some heavy metals in damaged soils from mining like Cu and Pb (Pardo et al, 2011, Robles-Gonzalez et al, 2008) and also accumulate redundant nitrates (de la Fuente et al, 2010, Allred et al, 2001).

As already mentioned, slurry can be also used as substrate for production of biogases like methane or H_2, or some alcohols, or fatty acids (Ocfemia et al, 2006). All these products are biofuels and can potentially replace conventional oil and gases in the 21st century in the industrial and domestic utilization. Their production is based on the fermentative activity of microbial and fungal microorganisms that are able to convert high-molecular components to low-molecular ones with a high yield. The process usually occurs in the same bioreactor conditions and sometimes is coupled with a fermentative decomposition so it can be a side product.

There is also a process is called thermochemical conversion (TCC) (He et al, 2001) and unlike of normal bioreactor decomposition this one requires higher pressure (7.5-10 MPa) and temperature (285°C) but faster in time (120 minutes) and is a fast pyrolysis (Serio et al., 2002). This method helps to convert biomass to liquid oil instead of ashes.

Additionally, the slurry decomposition can be combined with a formation to wetlands and a growth of some cultures like soya beans or rice. Although the yield of the grain is not as high as in the specialized methods of cultivation, this maneuver allows utilizing N and P and gets some extra place for a food production (Szogi et al, 2000).

2.4.3 Slurry production

Slurry is a side-product of waste management. Slurry can be made of manure or feces of animals and humans, rest of dairy products and wastes of biodegradable products. Except animal related sources, there can be involved the wastes of paper and forest industries. All there sources can be mixed or used as a monosource of carbon. Decomposing them into

slurry helps to remove such wastes out of the category of pollutants and transform them into something valuable. The process can involve air, such as activated sludge process, or be anaerobic in anerobic digestion technology. But the quantity of sludge that can be subjected to anaerobic conditions is less and should be divided to portions due to special equipment size.

Figure 10 represents the scheme of process that is used in waste treatment and leads to bio-slurries production.

All sources should be pre-treated to remove additional products, like lignin which inhibits microbial activity, and sieved to separate large or non-decomposed inclusions (plastic, metals). The process includes solids separation (is necessary to remove oils, grease, fats, sand, grit, and big solids), equalization (grinding and grating), neutralization of pH, aeration, settling, clarifying, chlorination. (Libhaber and Orozco-Jaramillo, 2012) That is why using if pig or cattle manure is so preferable – is requires only minoric pre-treatment. The waste treatment systems are built nearby to places of the major production of the wastes because it is economically disadvantageous to move the substrates further then 1 km (Kunz et al, 2009).

Slurry formation can be coupled with synthesis of manganese ammonium phosphate $MgNH_4PO_4 \cdot 6H_2O$ (MAP) in form of struvite crystals in production. Struvite is a very effective fertilizer and, in the same time, it can decrease amount of ammonia in the residuals and increase the recovery of phosphate from the system. This system needs some manganese addition and very sensitive to pH conditions. But it has very high fertilization perspectives and is sold, for instance, in MagAmp brand name. (Jaffer et al, 2002)

There are different kinds of bioreactors that involved in digestion of slurry. But normally they consist of several reservoirs and the slurry bioreactor is the main one and relates to batch or semi-continuous types of bioreactors.

First, swine and cattle manure are left in a regulating reservoir and after some time of explosion the manure comes to a bioreactor for digestion. Soil, additional nutrients, surfactants, and inoculums of digesting bacteria are added to the bioreactor as well. Usually empting of the bioreactor is not fully so the new portion is exposed to the old one, it make a uniform product. The digester tanks are very large – up to 1000 m^3 and 25 m in a diameter and are equipped with a gasometer and mixing machines to achieve homogeneity. From the bioreactor the slurry enters to a system of a reception tank and later to, for instance, oxidation tanks (ponds) sometimes with extra oxygen to the first stage or to the composting (Kunz et al, 2009).

Sometimes the bioreactor can be divided in two smaller ones so there is an option to change temperature or pH and so the microbial fauna of the mixture. It helps to increase rate of decomposition. Zhang and others in 2000 tested two systems: one mesophylic (35%) plus one thermophylic (55%) and two mesophylic (35%). The thermo-mesophylic pattern shows better performance - 6-15% increase of the removing of volatile solids. Another important feature of increasing temperature is decreasing of rate of *E.coli* in the system (Kudva et al, 1998).

After the anaerobic digestion the residuals can be dewatered and subjected to an oxidation process while the biomass after the similar dewatering process is used in a composting. After the activated sludge creation process, the residuals can be moved into polishing process, such as anerobic filters, to destroy the bacteria and remove some organic matter in efflux liquid, so the extra material can disposed in environment. While the biomass moves from the AS to the drying. (Libhaber and Orozco-Jaramillo, 2012)

Problem that is involved in decomposition of slurry is producing of some unpleasant odor. It caused mostly by sulfuric compounds: sulfuric (-S), mercaptane (-SH) and theophenes, and some others, like aromatic chemicals or methane. The simplicity of chemicals increases with time of decomposition. For instance, H_2S, COS, CS_2, CH_3SH have the most percentage in digested slurry (Clanton and Schmidt, 2000) but in fresh manure there are as well some higher-molecular weight compounds like thiophenes or thiocresols.

The removing of odor complainant sulfur substances can be achieved with different methods. The simplest is an adding of algae in the aerobic ponds which used energy of sunlight to reduce the odor (Gilley et al, 2000). It can be also a system of closed coupled anoxic and aerobic tanks and so the slurry moves from one to another in a cycle with all sulfur that synthesized during anaerobic condition to be decomposed and oxygenized in aerobic, without any emission to atmosphere (Pan and Drapcho, 2001). An ozonation allows rapidly remove the sulfur to less odoriferous from too, but this process is still under study (Wu et al, 1998). Another variant is the special constructed wetland tanks with a subsurface flow, so the major mechanism of removing compounds here are the mineralization and oxidation (Wood et al, 2000).

Oxidation ponds, in their turn, are exposed to oxygen supply because they are open. Such ponds are usually covered with geosynthetic clay liner (GCL) which protects the surrounded soils from contamination. The barrier function occurs because of the high turbidity in the inner layer of the liner in a contact to clay particles – betonite and salts in the slurry. Brown and Shakelford in 2007 have been tried to test these hydraulcal mechanisms within GCL and simulated animal waste solution. They found that turbidity is 4.2 times higher in aerobic conditions in slurry than in the water. (Brown and Shakelford, 2007)

After the decomposition the wastes can be additionally nitrificated, for instance. It can be used if the slurry is made from the paper or wood wastes, but not from the manure, where the nitro content is very high. The low levels of nitrates and nitrites are also caused by deficient of *Nitrosomonas* and *Nitrobacter* in the sludge. Their number increases during the aerobic digestion but not enough to reach a significant level and use all the ammonium in the system. Vanotti and Hunt in 2001 tested special nitrification pellets to determine if this method is more helpful. They use a special sludge accumulated in poly-vinyl polymers with entrapped large concentration of nitrifying bacteria. In combination with pH monitoring, it helps protect *Nirtomonas* from HNO_2 and so increase efficiency. This

method requires extra machinery but it allows removing for a half of NH_4^+ in the environment in a very short time period. (Vanotti and Hunt, 2001)

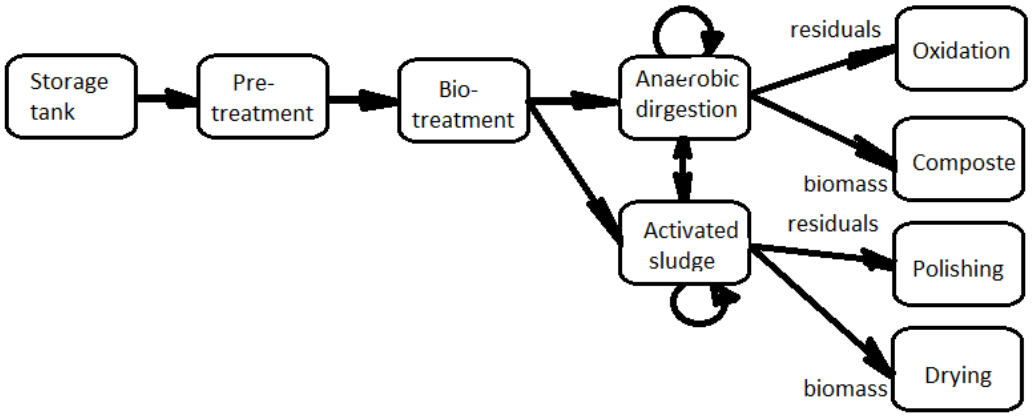

Figure 10. *Possible scheme of bioreactor for slurry treatment.*

3.0 Materials and Methods

3.1 Cell sensors and luminescence measurement

The slurries were tested with two methods – in dynamic and with the end-point. The end point was made for the antibiotic test and for the standard addition. It was performed as incubation in shaker with a sequenced single measurement in the Hidex Chamelion, Turku, Finland for luminescence counting. On the other hand, the dynamic was measured every several minutes with incubation directly in Hidex Chamelion, Turku, Finland.

If the cells are subjected to the testing in dynamic, there is a preheating of a plate without the cells is performed. The plate is left in shaker at 30°C or 37°C for 10 - 15 minutes to warm up the mixture and so the cells do not get into the difficult environment. It helps to make the response more stable in first several measurements.

All measurement tests were made in triplicates and the statistic was made to the induction factor (IF) evaluation and for the correcting the signal with the standard deviation. IF is a relation of the point result to the blank sample result. The blank result is a luminescence of the cells without heavy metal added (100 µl of the sensor cells + 50 µl of the slurry + 50 µl of MQ water). This allows to normalize the data and also makes it possible to compare them between each other – the obtained raw numbers of luminescence counts can differ very much simply due to difference in initial luminescence.

The reaction mixture composition is shown in the figure 16 below. On 96-well plate three samples and water are set in 3 columns each (Fig. 17). In this case, even if there is an overlapping of the signals from other sample row, at least the middle response can give the reliable response. Water also works as a control in each measurement. 8 rows allow making 7 dilutions and blank water in each measurement.

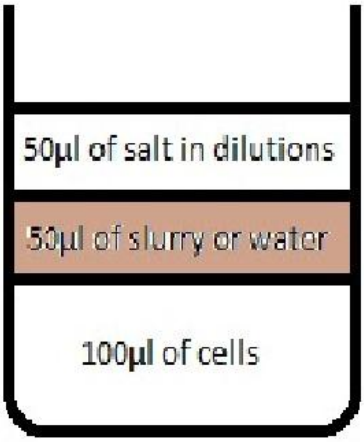

Figure 11. *Reaction mixture composition.*

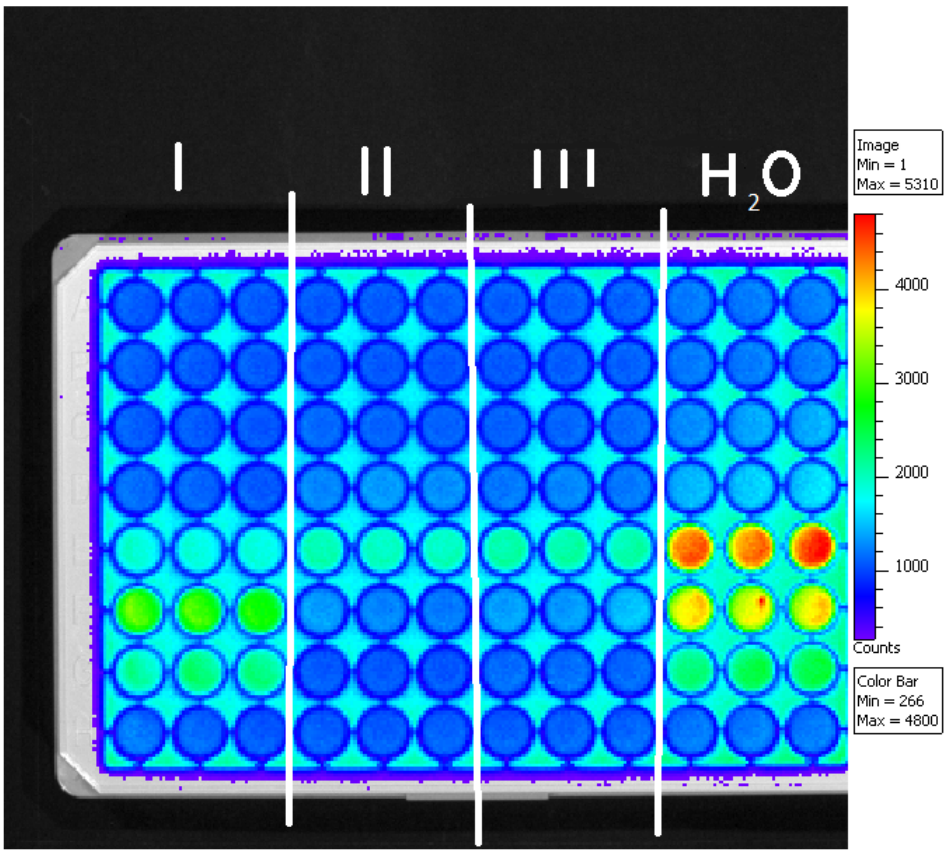

Figure 12. *Example of sample location on a 96-well plate made of filtrated 10% samples with mercury added after 1.5 hours at room temperature incubation.*

The additional data processing for combining all the curves in one was achieved with performing linear regression of the obtained curves on the stage of the fastest growth and then the incline was set with respect to concentration. Therefore the obtained curve represents the induction factor versus time and versus concentration and allows perceiving more information at one moment.

The error was combined out two sources – from the instrumental and from the pipetting and dilution making. The instrumental was obtained from standard deviation, while the pipetting was set as 5%. 5% was taken as systematical error because although the instruments were calibrated thoroughly, there always can be a risk of manual mistakes. The final error is a direct sum of these two.

For the measurements of the overall toxicity the control strain of *E.coli* MC1061 was used. The cells are got in a form of freeze-dried ampoules and revived in 1 ml of water for 2 hours at room temperature. Later the cells were diluted in deionized water to obtained desired amount. 100µl of the cell mixture and 100µl various concentrations (1%, 5%, 7.5%, 10%, 12.5%, 15%, 20%, 22.5%, 25%, 50%, 75%, 100%) of the slurries were used. First measurement was made in Victor^2, Perkin Elmer, USA after 2 hours of incubation in shaker

at 37°C, 300rpm. Induction factor versus concentration chart was plotted to determine the optimal concentration of the material in further experiments.

"Mercury" sensors *E.coli* MC1061 pmerRBlux were revived in 1ml of sterile double distilled (MQ) water with exposion at room temperature for 2 hours. Then the cells were dissolved in sterile MQ water 100 times and spread on LA plates with 100µg/ml of ampicillin. The plates were left overnight at 30°C and the luminescence was checked with the Xenogen, Perkin Elmer, USA and the Living Image@3.1 program. Several luminescent colonies were picked up to grow in liquid LB (Luria-Bertani) medium with 100µg/ml of ampicillin or in HMM medium with 0.05% of casein hydrolysate, 0.4% of glucose, and 100µg/ml of ampicillin. The tubes were incubated at 37°C, 300rpm overnight, OD_{600} in the morning was ~0.7. Luminescence of 100µl of the medium was checked via Victor. The mixture was diluted to luminescence about 1000rlu. Standard curve was made as adding of 50µl of HgCl2, Sigma-Aldridge, USA in different concentration to 100 µl of cell and 50µl of MQ water and incubated at 37°C, 300prm for 2 hours. The luminescence was checked. The successful clones were grown in a larger volume 50 ml of LB medium half diluted with sterile MilliQ water and 100µg/ml of ampicillin up to OD_{600} 0.7 at 37°C, 300prm. Then there was 50 ml of 20% of lactose added, so the final lactose concentration was 10%, and the cells were divided in 1 ml portions and freeze-dried in 48 hour cycle in liophilysed machine. The ampoules are stored at -80°C. The cells are also stored at -80°C in 25% glycerol.

The mercury testing assay was made for the $HgCl_2$, Sigma-Aldridge, USA and for Met-$HgCl_2$, Sigma-Aldridge, USA dilutions for 1 % or 10% of initial dilutions of the slurry. In order to get to 100µl of cells there are 50 µl of 2% or 20% of slurry for standard curve and 50µl of mercury salt (Fig. 16). The plates without the sensing cells were preincubated at 37°C, 300prm in shaker for 15 minutes and then incubated at 37°C for 2 hours in Hidex Chamelion, Turku, Finland with measurements for every 2,67 min.

"Lead" sensor, *Pseudomonas putida* K2431.2440 pDNPczclux1, had been kindly presented as living cells on LA plate from laboratory of Helsinki University by Dr. Marko Virta. Several luminescent colonies were picked up to grow in liquid LB medium with 12.5µg/ml of tetracycline and in Heavy Metals Medium (HMM) medium with 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline. The tubes were incubated at 30°C, 300rpm overnight, OD_{600} in the morning was about 0,45 in the HMM medium and 0.8 in LB. The colonies were tested on initial luminescence and also on Pb standard curve. The best responded colony was grown in larger volume – 50 ml (30°C, 300rpm) of HMM medium with 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline and later diluted in 10% lactose and freeze-dried in 48h cycle. The ampoules have been stored at -80°C. There is also an option to store the samples in glycerol dilution at -80°C but the cells are very sensitive to glycerol even in low concentration.

Standard curve to identify range of the cell work was made as adding of 50µl $Pb(NO_3)_2$ Sigma-Aldridge, USA, or $CdCl_2$ Sigma-Aldridge, USA, or $NiSO_4$ Sigma-Aldridge, USA,

or $ZnCl_2$ Sigma-Aldridge, USA in different dilutions, to 100 µl to cells and 50µl of water in different pH values. The plates without the cells were preincubated at 30°C, 300prm in shaker for 15 minutes and then and incubated at 30°C, 300prm for different time period. The luminescence was checked in Hidex Chamelion, Turku, Finland. The Pb and Cd standard curves give the lowest IF and require 8 hours, albeit the Zn tests gives the highest response after 3-4 hours of measurements. Nickel varies in time and response from sample to sample, so the 18 hours measurement with 3 hours of preincubation protocol was chosen.

For the direct test the cells were revived in 1 ml of MQ water and exposed for 2 hour at room temperature. The initial luminescence in this case is quite low so to increase amount of the cells, they was grown in double amount (2 ml) of water at 30°C, 300prm for 3-4 hours. After adding water, luminescence of 100µl of the revived cells was checked via Hidex Chamelion, Turku, Finland. The mixture was diluted to luminescence about 1000rlu.

Then the metal testing assays were made for the subjected salt concentration and 1 % or 10% of initial dilutions of the slurry. In order to get the experimental mixture to 100µl of cells there are 50 µl of 2% or 20% of slurry and 50µl of salt (Fig. 16). The plates without the sensing cells were preincubated at 30°C, 300prm in shaker for 10 - 15 minutes and then incubated at 37°C for 2 hours in Hidex Chamelion, Turku, Finland with measurements for every 8.34 or 16 min.

The antibiotic concentrations were determines with the *E.coli* pBLalux1 for ampiciline and *E.coli* ptetlux (Korpela M., et al 1998) for tetracycline. The sensors were obtained as freezed-dried ampoules and the cells were revived in MQ water for 2 hours and then the cells were exposed for the slurry or the antibiotics dulitions. The reaction was again made in the microtiter plate (100µl of the cells, 50 µl of MQ water and 50µl of 2% or 20% the slurry or the antibiotic). Unlike of the previous continuous measurement this particular experiment was made with respect only to the final point of the testing. The plate was incubated in shaker for 3 hours at 37°C, 300prm and then the measurement was made in the Hidex Chamelion Turku, Finland luminescent counter. The evaluation of the compound amount was made according to the standard curve.

The curves of IF versus time were transformed to tables which indicate their maximum point and an optional time-point of the inhibitory activity.

Also there are tables created on the end-point basis – the concentration of the metal ions was evaluated according to the final signals and the standard curve. Then the results were subjected to the original known concentrations added.

For more precise determination of the chemical compound concentration, the standard addition method was made. To the known and constant amount of the tested solution various increasing amounts of the standard solution with known concentration of salt are added. Then the tested samples are diluted up to the same volume with water (100 µl). Then 100 µl of the sensing cells was added. The responses of the samples are tested on luminescence.

The results are plotted in graph of the added heavy metal concentration with respect to the signal. Later the linear least squares analysis on the points on one linear plane is made

so the intercept with axis and slope are found. Through these numbers, the amount of the heavy metal initially contained in the system is found.

3.2 Slurry

The samples of slurry were taken in central and western Finland around Tampere. There were three samples subjected to these experiments: one from pig farm and two from biogas plant.

The pig farm sample is a raw sample (1st) that has not been in anaerobic digester but been stored in an open storage vessels with solid separation. So this slurry contains only poorly processed manure, some bacteria from intestine tract, and surfactants can be included only as washing liquids for the farm needs. The slurry was also transported to biogas plant (the 2nd and 3rd samples), additionally, it was used twice a year for local field fertilization too. In the digesting period the fresh manure is being added to the already digested one. Few days before sampling, there was a rain for several days and in the sampling period there were heavy snow showers.

The biogas plant performs two samples: before and after anaerobic digester. The biogas plant is filled with the material from 10-12 small-to-medium size pig farms around and the industrial and municipal biodegradable wastes. Some of the farm sent raw manure, and some make the prior solid separation. The wastes are tested beforehand, homogenized, all the solids are separated until about 10% solids left, some synthetic additives are used. Then the material was heated till 92°C for 2 h, digested in anaerobic digester (38°C, 6700 M^3) for an average of 18-23 days. After the digester, the slurries were centrifuged to remove the solids as much as possible, evaporated with heat, dried and stored before shipping. The rejected water with ammonia content was stripped in a stripping tower for ensuing land use for recycling of the water for solid dilution. The generated electricity is used for national grid.

Another group tested the samples for their own tasks on metals with atomic absorption microscopy, pH, alkalinity, surfactants, solids and amount of ammonia. The solid measurement was repeated in this work as well, due to long period of storage the slurries before starting the current experiments.

Solid measurement was made in for two types – the total solids (TS) and total volatile solids (TVS) according to the Methods 2540B&E. A sample of known volume (2 ml) was put in a preweighed aluminum dish (B) and first dried at 105 °C for 4 hours in a thermostatic condition, weighed again (A) after cooling down at room temperature in a dissector for at least 4 hours to avoid the moisturizing of the sample, and then burned down to ashes in muffle furnace at 550°C for 2 hours. After following cooling, as described above, the samples have been weighed repeatedly (C). All measurements have been made in triplicates. TS and TVS were calculated with the equations below (6) and (7). TS mean a weight of dried material in the slurry, while the TVS are recognized as TS without decomposed organic material. In this particular case solids are usually composed by clay, organic materials from the manure, and other small particles. In this study the samples

would be measured with and without the TS. The removing of the solids was made with filtration through 0,45 μm.

$$\text{TS (mg/ml)} = \frac{[A-B]*1000}{sample\ volume\ in\ ml} \quad (6)$$

$$\text{TVS (mg/ml)} = \frac{[A-C]*1000}{sample\ volume\ in\ ml} \quad (7)$$

Table 4. *The Total solids and total volatile solids data obtained soon after sampling.*

	TS	TVS
1st	10,4±0,3	5,0±0,04
2nd	76,1±0,1	27,5±0,2
3rd	41,2±1,8	16,7±0,0

pH was measured with WTW pH-meter, model pH 330i, with two pointed precalibration and temperature sensor. The other group found that aging does not effect on proton concentration. In this study pH of the slurry was adjusted before addition to sensors to avoid cell shock and unify the method.

Other important parameters that are tightly bound to pH are alkalinity and ammonia concentration. Alkalinity is ability of the solution to keep stabilized pH with adding H+. It is usually achieved with carbonate-bicarbonate system in the media. So it depends on dissolved carbonate and partial pressure of CO_2 in the environment. Other systems that involves in the ion exchange are organic and inorganic acids like nitrates, phosphates, or sulfides. The alkalinity was determined on basis of volumetric characteristics with sulfiriic acid with known concentration as standard solution and as a titrant according to potentiometric titration to preselected pH (2320 B.4.c, APHA, AWWA, WEF, 1999)the preselected point were 5.8, 5.3, 4.5, 4.3. Alkalinity determines the stability of the system and may refer to some condition that effect on the cells. All samples have very high alkalinity about 8000-9000 mg/L, while the 2nd one has extremely high potential to acid neutralization- about 47000 mg/L, so all the solutions are very stable in the case of adding acids or bases.

The dissolved ammonia is mostly origin from pig and cow manure and because the slurries, essentially the 1st one, are composed mostly of it, it can have an especially much influence. Ammonia NH_3 is toxic to most of living beings in a high concentration so its emission to atmosphere should be limited. But, on the other hand, ammonia can be converted to nitrates by some soil and root bacteria during the fertilizing process. Also NH_3 can affect the pH as well because in acid environment it can easily turns to NH_4^+ with binding of proton. The measurement was made with ammonia selective membrane electrode, Orion 95-12 connected to an Orion 290A meter. Ammonia amount is quite low <1g (NH_3-N)/kg wet, and the amount is increasing with time.

Surfactants can be sometimes added to digesters to increase the velocity of the decomposing and create one unify medium. But in case of 1st sample, it is primary the

cleaning liquids with that the farm removes manure from the property. Surfactants can effect on cell activity as well. The testing was made with Lange Test Kits (cationic – LCK kit 331, anionic – LCK kit 332, non-ionic ones – LCK kit 333) and HACH LANGE DR2800 spectrophotometer on multiply barcode modes. Results is that there is high amount of anionic surfactants in first sample and non-ionic in the 2nd and 3rd ones.

Heavy metals were tested with convenient methods of AAS the method that was described above. The measurement was made on a Perkin Elmer Spectometer (A-Analyst 400) (Fig. 13) in order to manufacturer protocols and methodology. The testing was made with specialized lamps Ni, Zn, and As with Lumina lamps and extra As EDL-Lamp. The solutions for establishing standard curve are commercially available and made every time prior analyzing. No extra addition was made to the described metals.

Figure 13. *Perkin Elmer Spectometer(A-Analyst 400)*

Hg, Pb, and As was also tested outside of the laboratory because the chemicals need special analytical tools and equipment and the lab was not satisfied to the conditions of measurements.

Table 5. *Heavy metal contents and pH of the slurries 1-3. (< = below detection limit)*

	1st	2nd	3rd
pH	7.7	8.3	7.0
Zn	6.8 mg/kg_{wet}	15.5 mg/kg_{wet}	18 mg/kg_{wet}
Ni	<0.1 mg/kg_{wet}	1.3 mg/kg_{wet}	1.3 mg/kg_{wet}
Cd	0.3 mg/kg_{dry}	<0.2 mg/kg_{dry}	0.1 mg/kg_{dry}
Hg	<0.07 mg/kg_{dry}	<0.07 mg/kg_{dry}	<0.1 mg/kg_{dry}
Pb	<2 mg/kg_{dry}	<2 mg/kg_{dry}	<2 mg/kg_{dry}

After sampling the materials were stored at 5 °C. The slurries were centrifuged at 10000 rpm, 15 min - these samples are called non-filtrated. The filtrated samples were further processed with reverse osmotic condition through consequent Watman filters of 47 mm, 5 mm, 6µm and 0.45 µm.

The digested samples have been digested in HNO_3, Sigma Algridge, USA and heated until a complete degradation of organic molecules according to AAS digestion protocol. The 50ml of the slurries was warmed up with 2 ml of 65% HNO_3 until almost dryness (1-2 ml left) and then extra HNO_3 was added in portions of 5 ml. The valuation if the material had been digested was made on basis of the fume color – when the fume color turned from orange-yellow to white, all the organic material is considered to be decomposed. Then the solutions was reconstituted in some amounts of double distilled (MQ) water and filtrated through 0.45 µm filter. The obtained amount was diluted to the initial 50ml volume in MQ water in volumetric flask.

Then the samples were diluted in MQ water for 2% and 20% and the PHs of the dilutions were adjusted to 6.9. The finally prepared samples are stored at +4°C for 1 week at maximum.

The filtration of sample is expected to remove all the particles and bacterial fauna of the slurry, so it allows decreasing of influence of ions in particles and concurrent growth of other bacteria. The digesting is needed for decomposition of all organic molecules that can bind ions or decrease activity of the sensor.

4.0 Results and Discussion

4.1 Cell tests and standard curves

The sample testing was tested in water with making the standard curves. The cells were obtained already transformed and tested in other papers but they were rather unreliable and instable. My primary task was to understand how they work and to increase the IF response if it is possible. In addition, the standard curves were needed to find out the range for further tests.

The problem of the obtained mercury sensor was that is had very low initial luminescence and there was not very high activity. Therefore, the cells were seeded on the LA plate and the most luminescented colonies (Fig. 14) were grew up in liquid LB medium with 100µg/ml of ampicillin. After repeating of the process there was a colony found that has the best luminescence. The clone was stored as freeze-dried in 10% lactose.

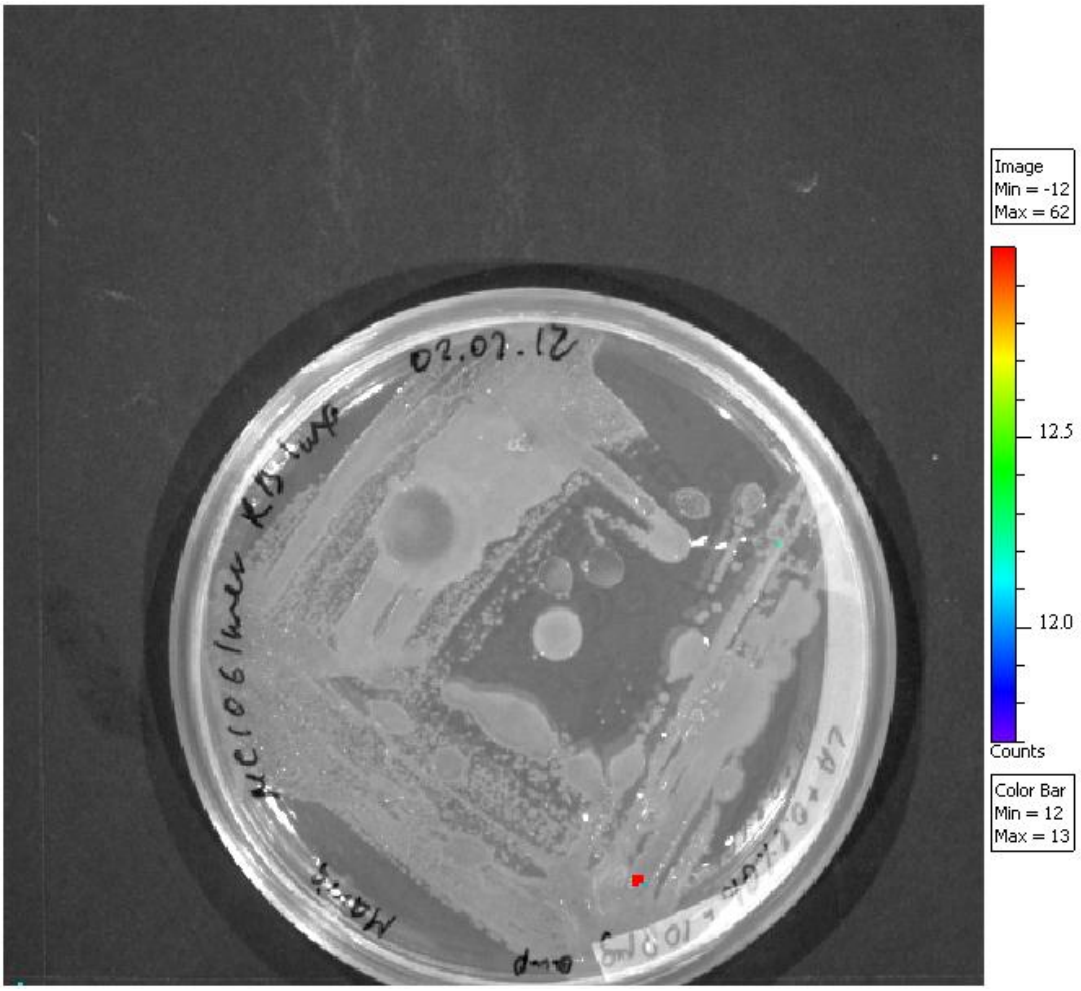

Figure 14. *Mercury sensor reseeded from an ampule. There was one clearly seen colony that subjected to further tests.*
Operation conditions: E.coli MC1061 pmerRBlux sensor strain after overnight incubation at 37°C, on LA plate with 100µg/ml of ampicillin.

The resulted colony was tested on mercury and methyl-mercury (Fig. 15). The obtained graph resembles the one in the article and shows high IF. Also there was a test if the concentration of the dissolved nutrients interferes with the response of the biosensors (Fig. 16). It is shown that the samples grown in LB/2 (equal mixture of water and LB) have higher IF than in one LB and in LB/8. As a result, it was decided to grow the cells in LB/2 medium and to store as freezed-dried ampoules in 10% lactose.

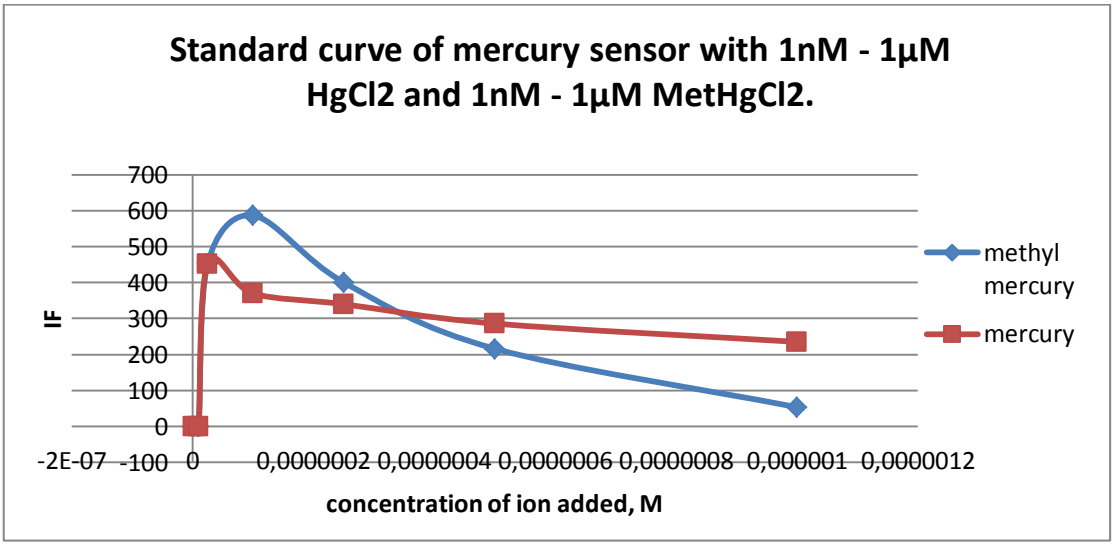

Figure 15. *Standard curve of mercury sensor with 1nM - 1µM HgCl2 and 1nM - 1µM MetHgCl2.*
Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, 300 rpm in shaker

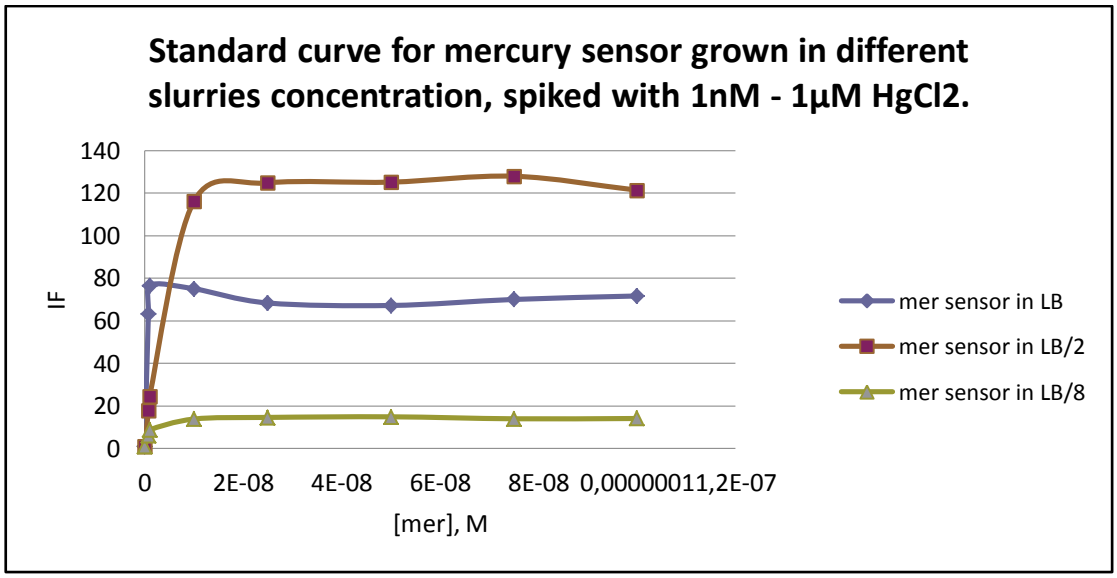

Figure 16. *Standard curve for mercury sensor grown in different slurries concentration, spiked with 1nM - 1µM HgCl2.*
Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, 300 rpm in shaker

The second sensor was tested on lead because, according to the study, the cells have the lowest response on it among all metals. The first attempts to check the sensor were failed. Cells had grown in LB medium with 12.5µg/ml of tetracyclin had high initial luminescence but the induction factor in the standard curve was about 1.3. The figure 17 represents the plate with the lead sensor – almost all cells have luminescence but the only difference that some of them have higher IF than other in the same conditions. The closer look to the article that described the sensors showed that there was a special growth medium used – the Heavy Metals Medium (HMM) with addition of glucose, caseine and tetracycline. But the cells did not give high response even in this case. The cells had initial luminescence around 1000 rlu, but there was no significant changing in ion additions. The testing was made for several pHs to determine if there was a problem. The HMM solution has pH 5 and the cells successfully grew in it but there was found that there was a quite narrow pH range for the Pb assay 6.7-6.9 (Fig. 18). The cells were adjusted to this pH after the growth but before the incubation. And then exposed to Pb assay. Another approach was to increase time of testing to more than 4 hours.

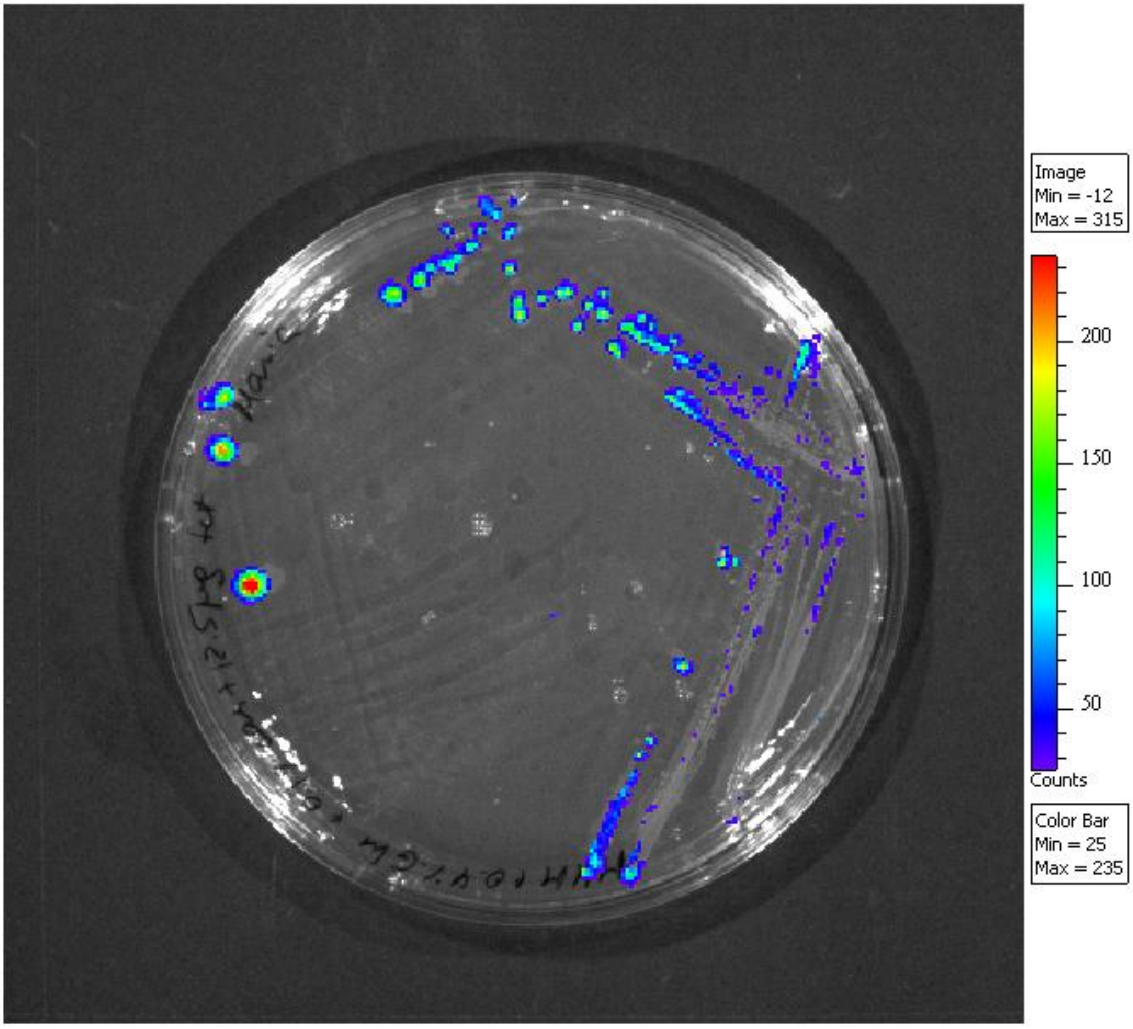

Figure 17. *Lead sensor reseeded from alive colony. All colonies have low luminescence.*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 on HMM medium with 1.5% agarose, 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline after overnight incubation at 30°C.

After lead, the cells were tested to zinc, nickel, and cadmium as well. It appears that the pH has broader range for these compounds but the 6.9 was chosen as a perfect one to adjust both the cells and the slurries. In case of zinc (Fig.19), there is clear rising of sensitivity with pH increasing – the higher the pH is, the less concentration the biosensors detect. Cadmium (Fig.21) and nickel (Fig.20) have similar pictures – the response is getting higher with less protons in the system.

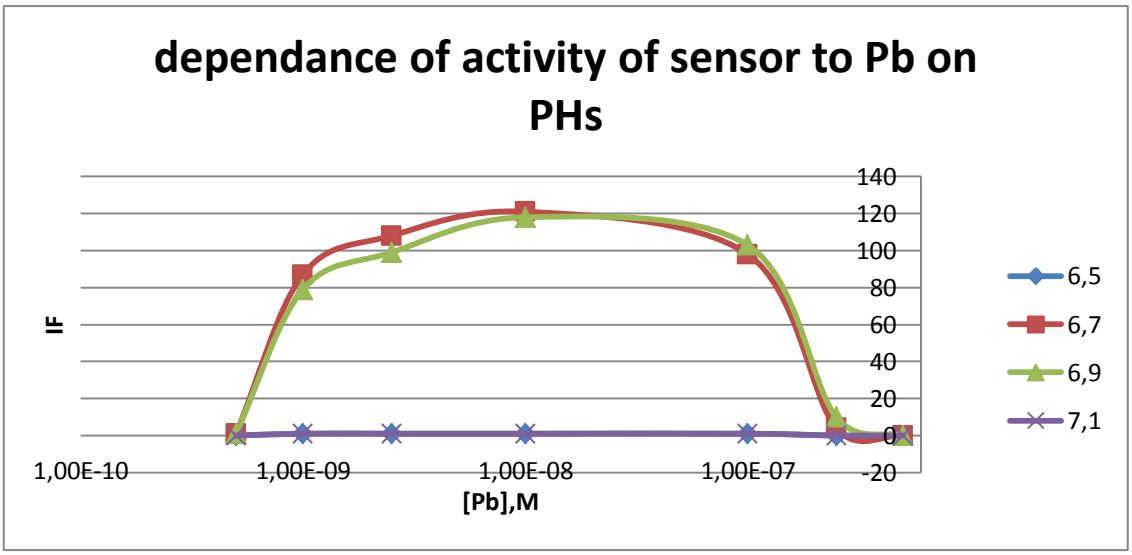

Figure 18. *Dependence of the activity of the lead sensor on lead ions at various pH Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1on HMM medium with 1.5% agarose, 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline after overnight incubation at 30°C, 300rpm*

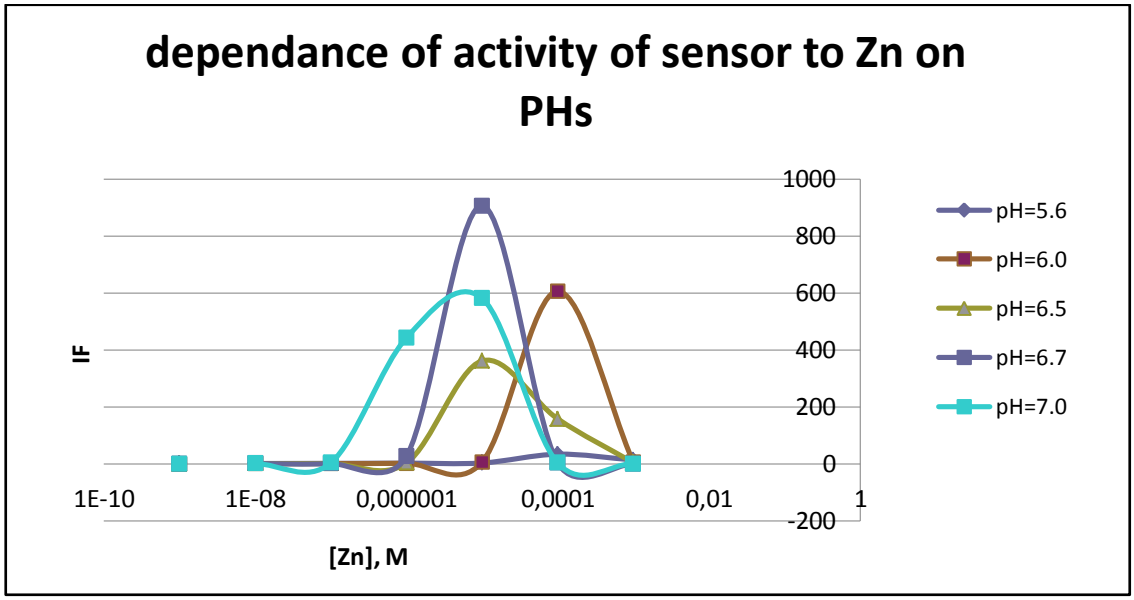

Figure 19.*Dependence of the activity of the lead sensor on zinc ions at various pH Logarithmic scale Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1on HMM medium with 1.5% agarose, 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline after overnight incubation at*

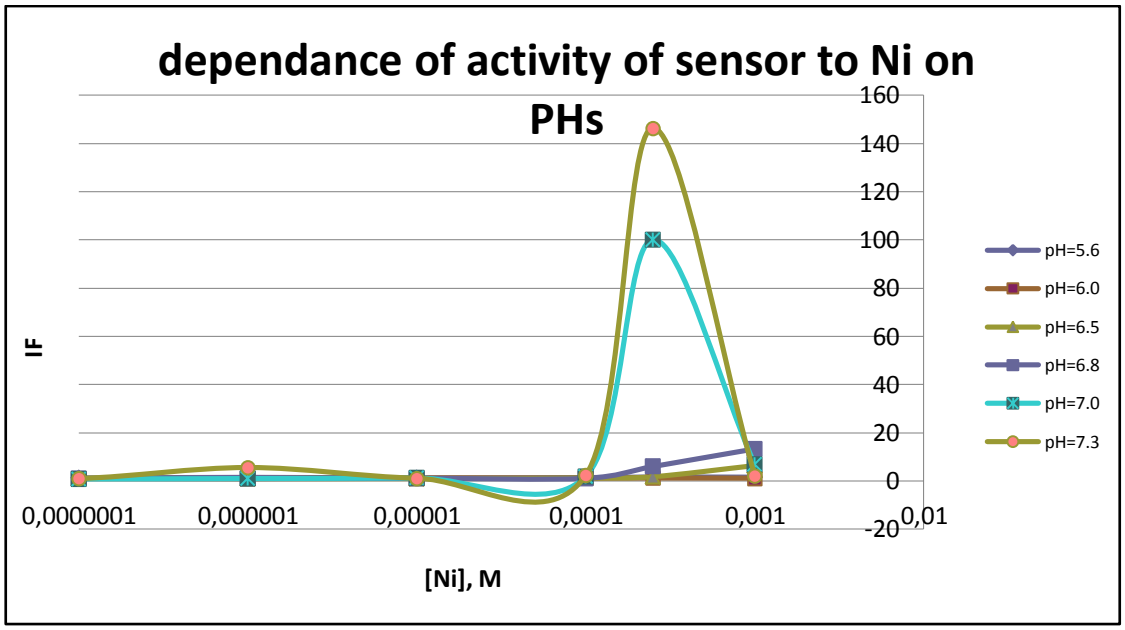

Figure 20.*Dependence of the activity of the nickel sensor on lead ions at various pH Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1on HMM medium with 1.5% agarose, 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline after overnight incubation*

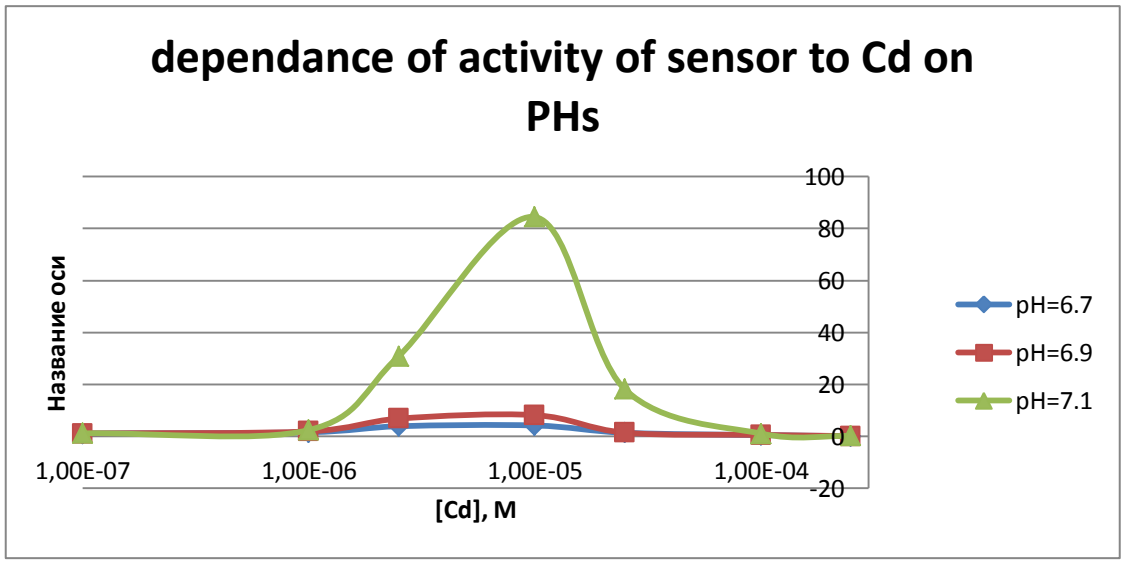

Figure 21.*Dependence of the activity of the lead sensor on cadmium ions at various pH Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1on HMM medium with 1.5% agarose, 0.05% of casein hydrolysate, 0.4% of glucose, and 12.5µg/ml of tetracycline after overnight incubation*

The experiment showed that there is the best time for adjustement of pH during liophilisation process is after the reaching the stationary phase and the adding the lactose

but before putting the cells to the liophilisation dryer machine. In all other cases the IF does not exceed 1.5 for lead.

Results of freezed-dried cells are normally lower because some cells died during liophylization and storage.

4.2 Solids

The solids in the non-filtrated samples were tested before providing the slurries to the further investigation. The purpose of the experiment was to predict the behavior and to evaluate how much of the sample is in the particles that can effect on the cells. Both total and total volatile solids were tested to check what percent of organic molecules represent the slurry. Obtained results are showed in table 6. In comparison to table 4 these numbers are lower, especially in 2nd and 3rd slurry. One of the possible reasons of such a big difference is a long resting of the samples – for a couple of weeks the slurries were in big vessels at +5°C before they were taken to these tests. Thus, part of the dissolved particles can settle down and not pour out during collecting the material for these tests.

Table 6. *Total solids in slurries*

	Total solids, g/l	Total solids, %	Total volatile solids, g/l
1st slurry	8	0,8	3,5
2nd slurry	14,7	1,59	6,4
3rd slurry	14,6	1,56	4,1

4.3 Overall toxicity

The overall toxicity was performed to evaluate the maximum concentration that can be used for the biosensors testing without the cell growth depressing. The slurries can contain some macromolecules, such as sulphur compounds or just a high concentration of ammonia that can kill quite sensitive cell lines like lab strains of *E.coli* or *P.putida.*

The testing was made as end-point measurement in rather high spectrum of concentration (Fig. 22) – was used a scale of 0 (blank), 5%, 7.5%, 10%, 12.5%, 15%, 20%, 22.5%, 25%, 50%, 75%, and 100%. 1st sample has lower toxic activity than the 2nd and the 3rd ones - the curve is higher.

After test of the different dilutions, the concentrations higher than 15% are considered as not-efficient so the points of 1 and 10% have been chosen. The 1% is chosen for evaluation of the slurry itself and the 10% can show a partial influence of the particles.

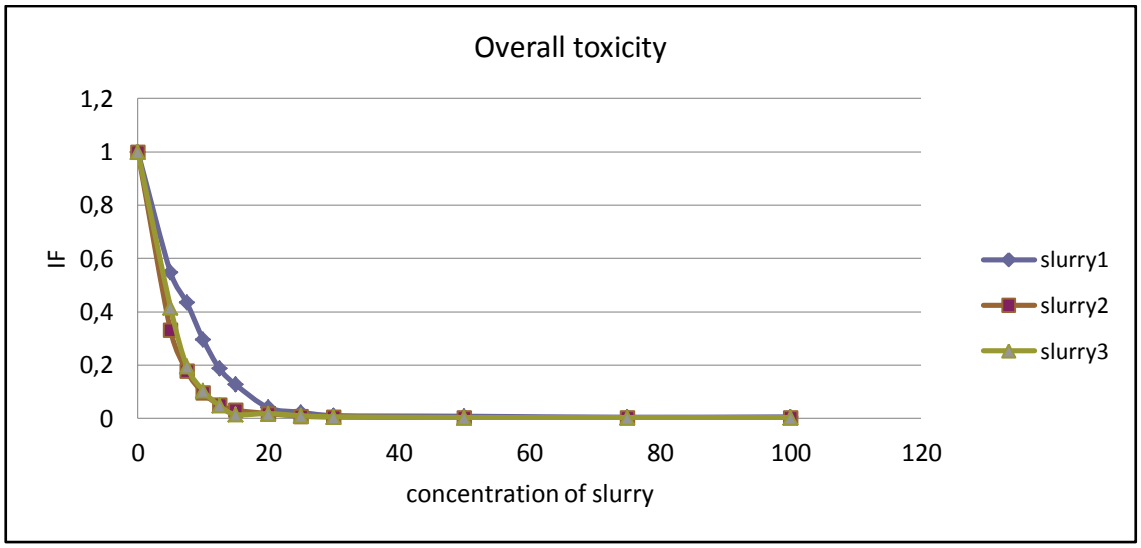

Figure 22. *Graph of overall toxicity of all samples in 5-100% range tested with end-point.*
Operation conditions: E.coli MC1061 with 2 hour incubation at 37°C, 300rpm. Tests were made in Victor2, Perkin Elmer, USA

4.4 Antibiotics

The tests on tetracycline and ampicillin were chosen because these are the antibiotics that are used in selective media for the biosensors growth. Table 7 shows that amount of the antibiotic is in normal value – very low, so there is very low probability that the antibiotics can cause harm during the fertilization. One of the probable reasons of such low values is that the slurries were subjected to short-term heating to 95°C so high-molecular compounds, such as antibiotics, has partially decomposed.

Table 7. *Concentration of antibiotics in the slurries.*

	Ampicillin	Tetracycline
1st slurry	0.4µg/ml	0.2 ng/ml
2nd slurry	0.78 µg/ml	0.5 ng/ml
3rd slurry	1.57 µg/ml	0.5 ng/ml

Operation conditions: E.coli pBLalux1 and E.coli ptetlux sensor strains with incubation at 37°C, 300rpm for 3 hours. Tests were made in Hidex Chamelion

4.5 Amount of heavy metals in the samples

It can be some mistakes in comparison of the results obtained from kg of matter (Table 5) and liters of liquid (Table 8) and referred to bioavailable or the total concentration. But it is seen a tendency that the 3rd sample performs more mercury or its accessibility is better to the biosensors.

Lead concentration is also very low and below the detection limit, but there is more of it in the 1st sample. Also the amount of lead is nearly equal in 2nd and 3rd slurry.

Amount of bioavailable zinc is higher in the sample from the digested tank. While the amount of the total zinc is seems to be higher in the 3rd sample.

Amount of cadmium resemble the obtained data – 1st slurry has the most and the 2nd and the 3rd has comparable amount.

Bioavailable nickel part is higher in the 1st slurry (26 μg/l out of <0.1mg/kg) than in the 2nd and the 3rd samples (~80 μg/l out of the 1.3 mg/kg)

Table 8. *Summary of heavy metals concentration in the slurries*

	1st, μg/l	2nd, μg/l	3rd, μg/l
Mercury	0.012±0.001	0.012±0.001	0.1±0.005
Methyl mercury	0.003±0.00005	0.003±0.00005	0.006±0.0001
Lead	4.57±0.21	0.05±0.002	0.82±0.04
Zinc	90±4	250±11	70±3
Cadmium	1.09±0.05	0.02±0	0.03±0
Nickel	26±1	83±4	74±3

4.6 Metal addition

4.6.1 Mercury

Figure 23 represents how the luminescence changes in time during exposition with mercury ions. The picture was taken in Xenogen, so the signal is rather low-the plate was incubated at room temperature without shaking.

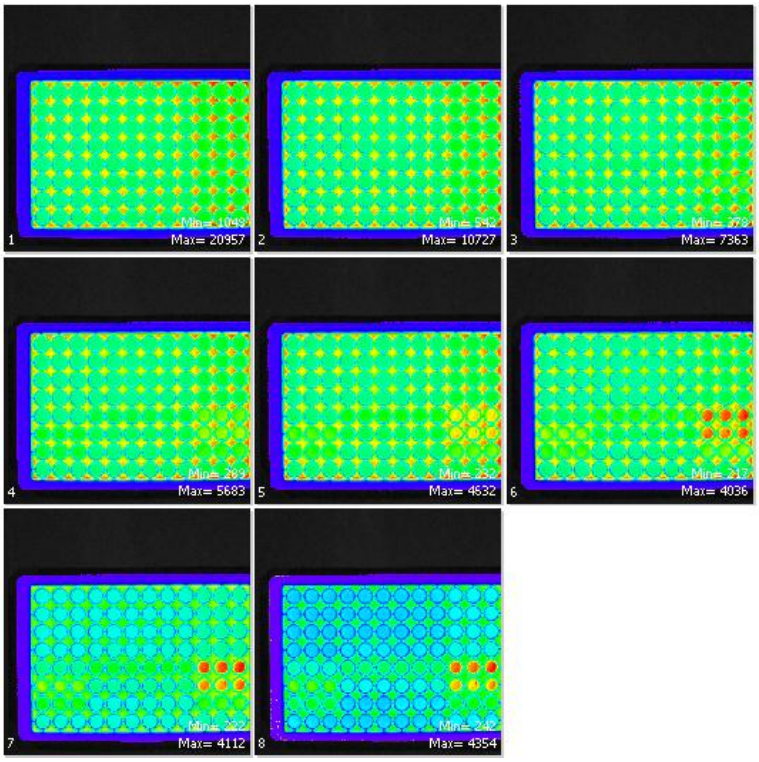

Figure 23. *Visualization of luminescence of filtrated 10% slurries 1-3.*
Operation conditions: E.coli MC1061 pmerRBlux sensor and 1nM - 1µM HgCl2 additions with counting every 10 minutes, incubation at room temperature in Xenogen

Table 9 suggests that the dissolved molecules decrease the mercury penetrating ability or have some inhibitory effects, for instance, the particles suppress growth of bacteria in media. Albeit in high concentrations, the nonfiltrated slurries are more reactive, so the particles protect the cells in high concentrations. Nonfiltrated 1% sample has the fastest activity among all – the ions penetrate the cell wall very rapidly and then the abrupt decline follows. Nonfiltrated10% sample, in the opposite, perform rather slow and steady growth and it is easily tolerates high concentrations of mercury in media. Digested samples are more sensitive to high concentrations and the lag phase is completely absent in their cases. One of the possible reason of this phenomenon is a complete disruption of organic in the

matter that can prevents cell grow or the ions consumption. Filtrated samples show better response in lower concentrations but it decreases after 250nM.

Table 9. *Summary of the slurries behavior with mercury addition.*

	1nM	10nM	25nM	100nM	250nM	500nM	1µM
1st filt 1%	2.1*	8*	50*	275*	390*	351*	193*
1st filt10%	1.5*	3.1*	19*(50 ●)	120* (50 ●)	310* (55 ●)	314* (50 ●)	169* (44 ●)
1st nonfil1%	1.5*	3.0*	31*	280* (40 ●)	520* (32 ●)	618* (22 ●)	557* (23 ●)
1st nonfilt10%	1.2*	1.2*	1.2*	6*	94* (48 ●)	381* (40 ●)	783* (34 ●)
1st dig 1%	1.8*	7.8*	47*	120*	81*	3*	1.5*
1st dig 10%	2.9*	9.4*	71*	85*	16*	4*	1.5*
2nd filt 1%	2.5*	11.5*	76*	335*	319*	120*	37*
2nd filt10%	1.9*	6.2*	39* (72 ●)	194* (55 ●)	200* (63 ●)	54*	14*
2nd nonfil1%	1.6* (30 ●)	3.8* (55 ●)	36* (40 ●)	290* (33 ●)	492* (36 ●)	563* (22 ●)	497* (22 ●)
2ndnonfilt10%	1.2* (40 ●)	1.8*	2*	43*	99*	208* (71 ●)	109* (46 ●)
2nddig 1%	1.5*	9*	41*	78*	47*	6*	2*
2nd dig 10%	1.7*	6*	74*	199*	15*	1*	0.2*
3rd filt 1%	1*	16*	71*	320*	292*	199*	89*
3rd filt10%	0.8*	11*	38* (42 ●)	162* (42 ●)	199* (53 ●)	42*	1.3*
3rd nonfil1%	0.5*	4*	26* (40 ●)	234* (36 ●)	536* (36 ●)	620* (38 ●)	547* (36 ●)
3rd nonfilt10%	0.2*	1*	5*	10*	54*	200* (48 ●)	271* (56 ●)
3rd dig 1%	125*	95*	62*	41*	32* (42 ●)	1.2*	0.2*
3rd dig 10%	0.6*	8*	42*	203*	16*	1*	0.2*
water	2.1*	16*	120*	354*	438*	468*	413*

** = highest IF in sample, ● = time in minutes when the inhibitory activity begins.*

Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

Combination of all curve slopes of the 1st slurry with mercury addition is represented on figure 24. It indicates that the responses of all the samples are lower than the response of water. The digested samples have highest sensitivity. But the responses of the nonfiltrated ones have the highest values.

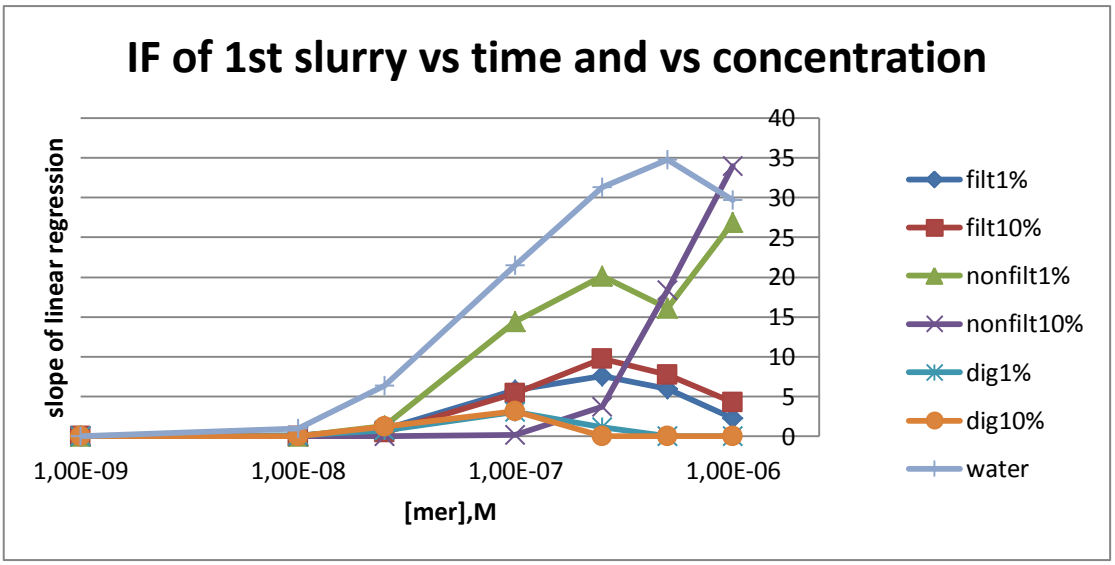

Figure 24. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs concentration of 1st slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

Figure 25 is a combination of all the linear regressions of the 2nd slurry curves. The nonfiltrated 1% of the 2nd slurry has the highest values and its pattern differs a lot from the rest of the samples, which do not exceed 10. Another difference is that the sample has higher sensitivity in comparison to the 1st slurry.

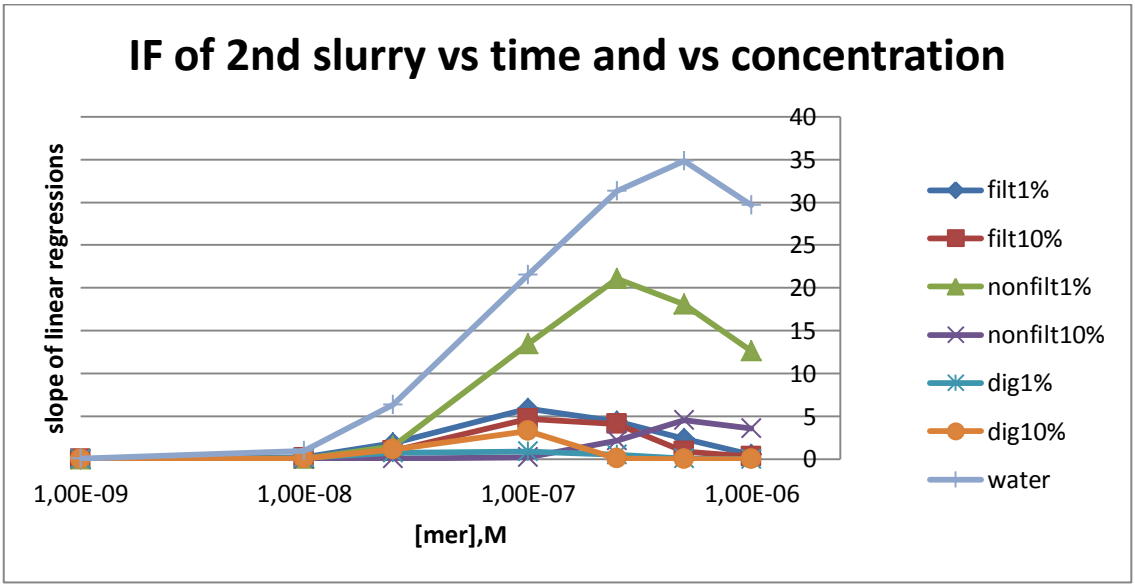

Figure 25. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs mercury concentration of 2nd slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

Combination of linear regression slopes of all the dynamic curves of 3rd slurry is shown on the figure 26. The pattern of the curves more closely resembles the pattern of the 2nd slurry with the sensitivity, but the values are relatively higher.

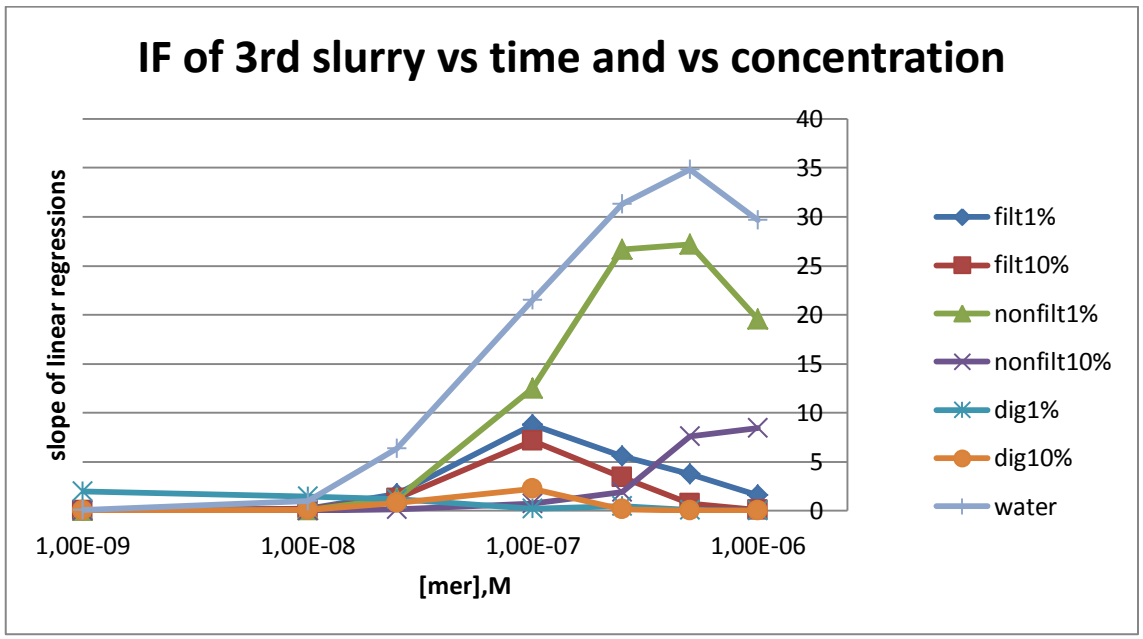

Figure 26. *Combination of linear regression slopes of all the dynamic curves IF vs time vs mercury concentration of 3rd slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

To evaluate the biosensor feasibility, the amount of known mercury added was compared to the responses of the slurries (Table 10). Concentrations less than 50nM perform responses corresponded to lower concentrations on the standard curve (for example, addition of 50nM returns value of 25nM maximum). The responses of 10% do not indicate the actual amount at all – it is very low. According to the table, the best choice of dilution and pretreatment combination could be the filtrated 1% - these samples have the closest responses to added amount.

Table 10. *Comparison of the actual amount of mercury with the amount evaluated by standard curve.*

	1nM	10nM	50nM	100nM	250nM	500nM	1μM
1st filt 1%	<1nM	3nM	10nM	60nM	n/d	100nM	1μM
1st filt10%	<1nM	<1nM	1nM	25nM	100nM	n/d	650nM
1stnonfil1%	1nM	1nM	11nM	25nM	50nM	>1μM	>1μM
1stnonfilt10%	1nM	1nM	1nM	25nM	75nM	100nM	125nM
1st dig 1%	1nM	2.5nM	12.5nM	75nM/ 350nM*	600nM	1μM	>1μM
1st dig 10%	<1nM	1nM	10nM	15nM	250nM	1μM	1μM
2nd filt 1%	<1nM	5nM	25nM	100nM	630nM	>1μM	>1μM
2nd filt10%	<1nM	1nM	2.5nM	50nM	100nM	800nM	1μM
2nd nonfil1%	1nM	1nM	10nM	45nM	50nM	>1μM	>1μM
2ndnonfilt10%	1nM	1nM	10nM	25nM	75nM	100nM	>1μM
2nddig 1%	1nM	2.5nM	12.5nM	40nM	50nM/ 600nM*	700nM	1μM
2nd dig 10%	<1nM	1nM	10nM	n/d	350nM	500nM	1μM
3rd filt 1%	<1nM	5nM	25nM	90nM	600nM	850nM	>1μM
3rd filt10%	<1nM	1nM	10nM	50nM	50nM	88nM	250nM
3rd nonfil1%	1nM	1nM	10nM	45nM	50nM	>1μM	>1μM
3rdnonfilt10%	1nM	1nM	10nM	30nM	60nM	75nM	100nM
3rd dig 1%	1nM	5nM	6.5nM	95nM	950nM	1μM	>1μM
3rd dig 10%	<1nM	1nM	10nM	n/d	350nM	500nM	1μM

n/d indicates response higher than that of blank. indicates two possible values on the standard curve corresponded to the obtained response*

Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

4.6.2 Methyl mercury addition

Addition of 1μM of methyl-mercury to 3rd slurry is shown on the figure 27. Filtrated 1% just increases the response up to IF 1900 in the peak. It is the highest IF response that was obtained in this study. Digested 10%, on the other hand, is continuously rising up to IF 857 without presence of inhibitory activity. Nonfiltrated 10% sample, in its turn, perform a plato-like pattern of the curve with stabilizing at IF 542 and later decreasing to IF 481. Responses of other samples are high but they are already in the inhibitory phase and decrease their IF in comparison to the previous amount of methyl mercury added. Filtrated 10% reaches IF 96, nonfiltrated 1% has IF 120, and the digested 1% is IF 184.

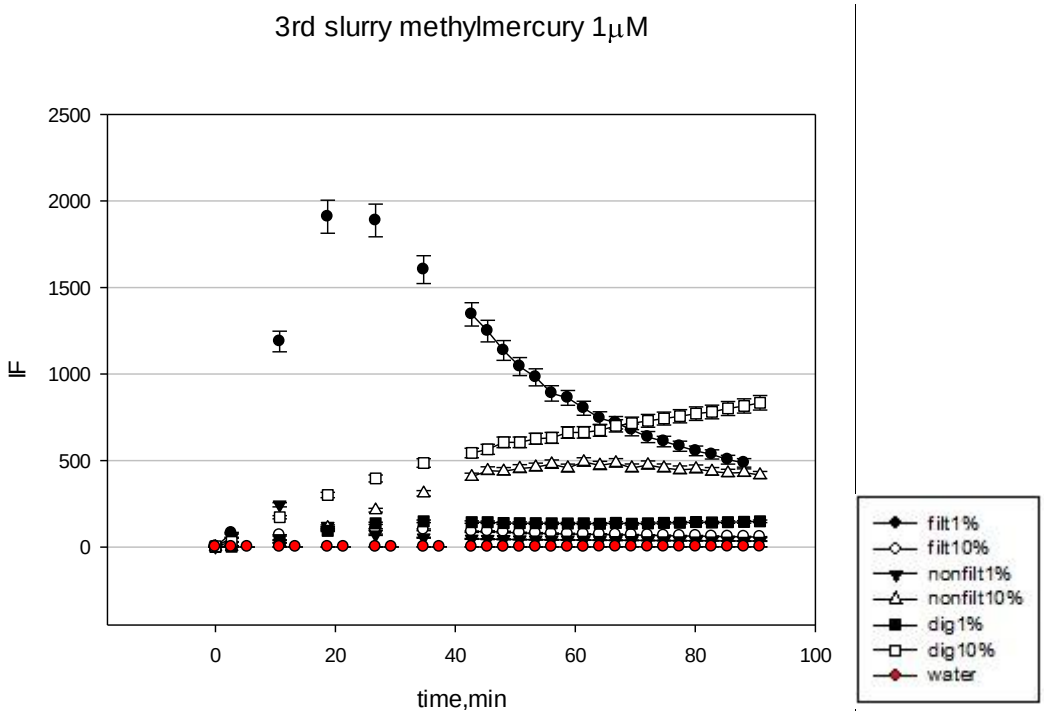

Figure 27. *Dynamics of 3rd slurry nonfiltrated 1% and 10%, filtrated 1% and 10%, digested 1% and 10% with 1μM MetHgCl2 added.*
Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex

The behavior of the sensor is more distinct and rough than in mercury addition. The graphs indicate slower passage of the ions through the cell walls – the lag phases are relativel;y longer. But, on the other hand, the presence of the organic compounds protects the cells at higher concentrations as it was shown in the mercury case. In comparison to the water curve, the dissolved compounds repress the biosensor activity at concentrations lower than 250μM. Concentration between 100nM and 250nM become inhibitory for most of the samples, except the digested ones. The highest responses, but in the inhibitory hump, are shown with the filtrated 1% samples with 1μM of methyl-mercury.

Table 11. *Summary of the slurries behavior with methyl mercury addition*

	750pM	1nM	10nM	25nM	100nM	250nM	1μM
1st filt 1%	1.3*	1.8*	1.9*	2*	34*	795* (23●)	1115* (19●)
1st filt10%	1.2*	1.4*	1.9*	43*	52*	85* (42●)	159* (24●)
1st nonfil1%	1.2*	1.6*	2.3*	625* (33●)	72* (23●)	784* (19●)	562* (19●)
1st nonfilt10%	1.4*	1.9*	3*	3*	101*	503* (42●)	798* (42●)
1st dig 1%	4.6*	6.3*	16.1*	41*	291*	254*	69*
1st dig 10%	8.3*	21*	24*	224*	294*	315*	360*
2nd filt 1%	0.8*	1*	1.7*	1.3*	17*	632* (24●)	1194* (19●)
2nd filt10%	0.9*	1.4*	1.9*	1*	17*	47*	101*
2nd nonfil1%	0.6*	1*	1.4*	21*	19*	216* (12●)	271* (15●)
2nd nonfilt10%	0.9*	1.5*	2*	86*	201*	403* (78●)	628* (74●)
2nddig 1%	1.5*	2.5*	3.5*	4*	403*	461*	425*
2nd dig 10%	1.2*	3*	6*	278*	287*	296*	464*
3rd filt 1%	1.2*	1.3*	1.5*	51*	147* (80*)	1478* (24●)	1942* (19●)
3rd filt10%	1.4*	1.7*	2.2*	5.2*	19*	29*	96*
3rd nonfil1%	1.5*	2*	2.7*	19*	23*	244*	120*
3rd nonfilt10%	1*	1.1*	1.8*	21*	96*	400* (71●)	542*
3rd dig 1%	101*	125*	237*	564*	573*	1086* (34●)	184*
3rd dig 10%	8*	10*	22*	289*	498*	572*	837*
water	30*	114*	141*	319*	474*	21*	5*

.

* = *highest IF in sample,* ● = *time in minutes when the inhibitory activity begins. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.*

Figures 28, 29, and 30 are combinations of the linear slopes and indicate that addition of the slurries make the cells less receptive to methyl mercury in media. Possible reason is that the macromolecules are easy to bloke the organic mercurials in the penetration phase. On the other hand, increasing of particle concetration creates better conditions for cells – the inhibitory activity of the nonfiltrated 10% slurries start later, so the particles can aggregate some ions onto the surfaces.

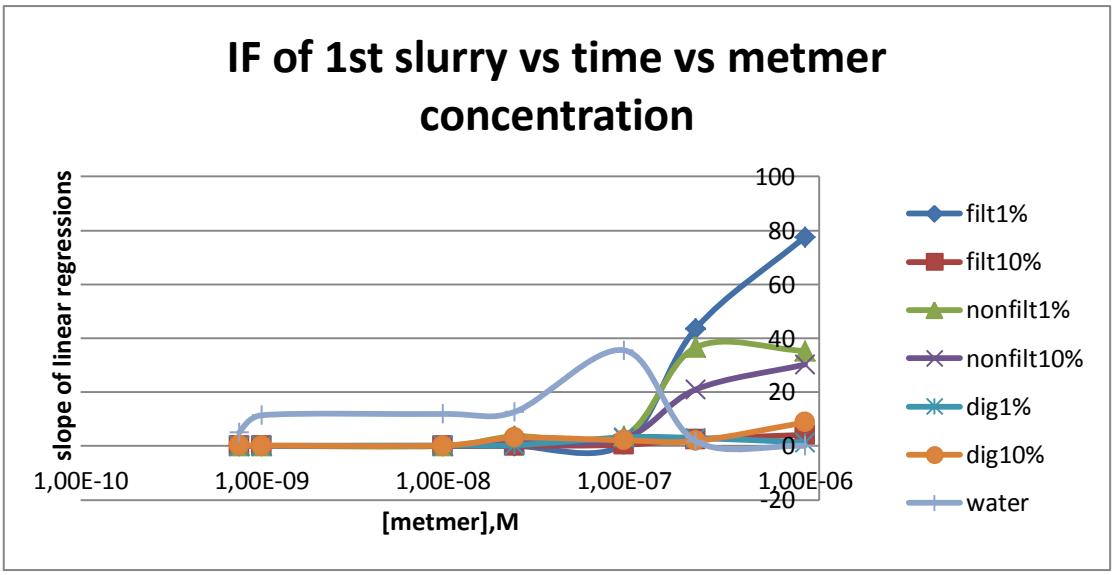

Figure 28. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs methyl mercury concentration of 1st slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

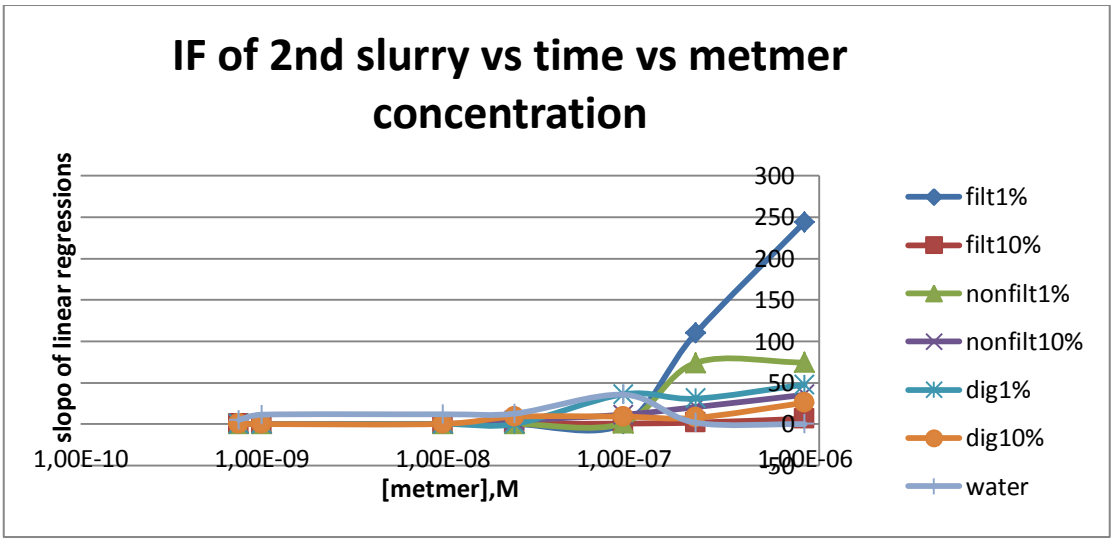

Figure 29. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs methyl mercury concentration of 2nd slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

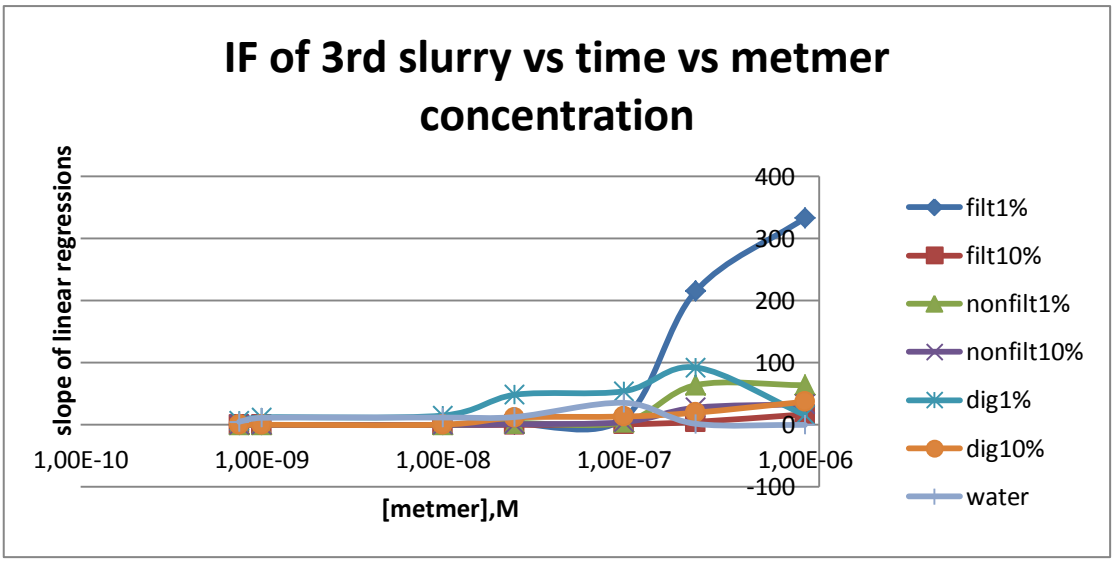

Figure 30. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs methyl mercury concentration of 3rd slurry.*
Logarithmic scale. Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex.

None of the samples returns the response comparable to the methyl mercury added. For the samples, obtained from the anaerobic digestor (the 2nd and the 3rd) filtrated 10% combination is a possible choice for testing methyl-mercury concentration lower than 50-100nM.

Table 12. *Comparison of the actual amount of methyl mercury with the amount evaluated by standard curve.*

	750pM	1nM	10nM	50nM	100nM	250nM	1μM
1st filt 1%	<750pM	<750pM	<750pM	2nM	50nM	150nM	150nM
1st filt10%	<750pM	<750pM	<750pM	15nM	25nM	40nM	40nM
1st nonfil1%	<750pM	<750pM	<750pM	1nM	1nM	10nM	10nM
1st nonfilt10%	<750pM	<750pM	750pM	10nM	50nM	100nM	110nM
1st dig 1%	<750pM	<750pM	<750pM	<750pM	5nM	8nM	180nM
1st dig 10%	<750pM	<750pM	750pM	30nM	30nM	30nM	30nM
2nd filt 1%	<750pM	<750pM	<750pM	1nM	12nM	150nM	150nM
2nd filt10%	<750pM	<750pM	<750pM	15nM	25nM	40nM	40nM
2nd nonfil1%	<750pM	<750pM	<750pM	750pM	750pM	1nM	1nM
2ndnonfilt10%	<750pM	1nM	10nM	50nM	100nM	110nM	200nM
2nddig 1%	<750pM	<750pM	<750pM	<750pM	75nM	100nM	100nM
2nd dig 10%	<750pM	<750pM	<750pM	25nM	25nM	30nM	50nM
3rd filt 1%	<750pM	<750pM	1nM	50nM	75nM	200nM	200nM
3rd filt10%	<750pM	<750pM	<750pM	15nM	25nM	40nM	40nM
3rd nonfil1%	<750pM	<750pM	<750pM	750pM	750pM	800pM	800pM
3rdnonfilt10%	750pM	750pM	10nM	10nM	50nM	110nM	110nM
3rd dig 1%	750pM	3nM	8nM	n/d	n/d	n/d	170nM
3rd dig 10%	<750pM	<750pM	<750pM	50nM	n/d	n/d	n/d

n/d indicates response higher than that of blank.

Operation conditions: E.coli MC1061 pmerRBlux sensor strain 2 hours of incubation at 37°C, in Hidex..

4.6.3 Lead addition

The bivalent metals were measured with another sensor type: another combination of the host bacteria and the plasmid, and so expected that it will perform another response pattern and activity in the slurry matrices. During the standard curve tests, lead response was the lowest one – nearly 15 after 15 hours of incubation so the 8 hours with each 15 minutes measurement protocol was chosen. Additionally, the digested 10% sample gave no response with the biosensor neither with lead nor with other bivalent chemical.

With 1nM lead ions added (Fig. 31) all samples exceed water response. Both 10% samples clearly repeat the inhibitory pattern with rising and decreasing. The cells have response IF 29.5 for filtrated10% and IF 10 for nonfiltrated10% in the highest point. Other samples are more stable in these conditions: digested 1% and filtrated 1% reach almost IF 15, and the nonfiltrated 1% - IF 5.

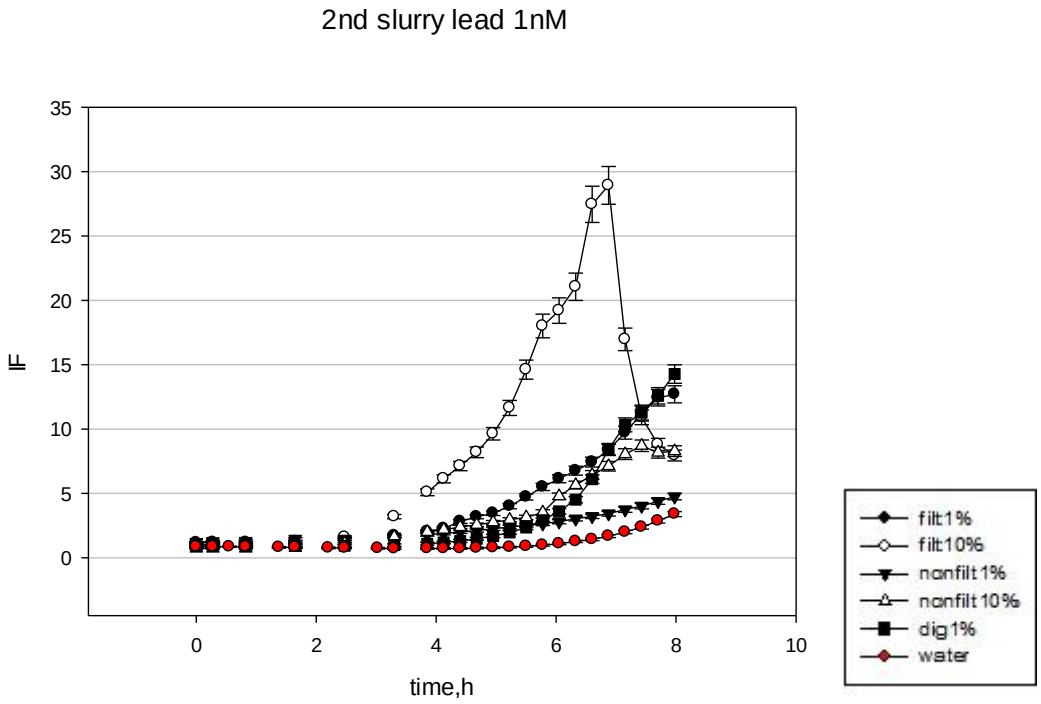

Figure 31. *Dynamics of 2nd slurry nonfiltrated 1% and 10%, filtrated 1% and 10%, digested 1% with 1nM $Pb(NO_3)_2$ added.*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

Normally the responses at some point exceed the water one. Not all the samples react well on the lead addition, but it appears that the 10% concentration of slurry shows higher activity than the 1%. It can be because the dissolved chemicals increase penetration of ions. The activity of all the samples starts at 100pM, while the nonfiltrated 1% sample reaches IF 4-5 only at 1nM lead concentration. The 1st slurry returns the highest response but, on the other hand, 100nM concentration inhibits its activity stronger.

Table 13. *Summary of the slurries behavior with lead addition*

	50pM	*100pM*	*1nM*	*10nM*	*50nM*	*100nM*	*250nM*
1st filt 1%	4.7*	11.5*	14*	14*	4*	1*	0.2*
1st filt10%	2* (7●)	28* (7●)	46* (7●)	41*	57*	97*	1*
1st nonfil1%	1.1	2*	4*	5.1*	3.5*	6*	6*
1stnonfilt10%	2.7* (7●)	31*	120*	8* (3.5●)	9* (3.5●)	14* (3.5●)	0.2*
1st dig 1%	1.9*	10*	11*	4.9*	3*	2*	0.1*
2nd filt 1%	2.3*	12*	13*	11*	10.5*	13*	2.6*
2nd filt10%	2.5* (6●)	10* (6●)	29* (6.5●)	28* (6.5●)	12* (6●)	6* (6●)	3.1* (6●)
2nd nonfil1%	1.2*	1.4*	5*	5.5*	2.1*	2*	7*
2ndnonfilt10%	1.5* (6●)	7.5* (7.5●)	8* (7.5●)	10*	19*	1.9*	4* (7●)
2nddig 1%	1*	8*	14*	13*	13.5*	5*	0.1*
3rd filt 1%	2.5* (7.5●)	9.4* (7.5●)	10.2* (7.8●)	8.9* (7.6●)	10* (7.6●)	10.3* (7.8●)	13.1*
3rd filt10%	1.8* (6●)	7.8* (6●)	26* (6.2●)	7.7* (7.2●)	4.9* (6●)	3* (6●)	2* (6●)
3rd nonfil1%	1.2*	1.7*	5*	6*	2.5*	2.8*	8*
3rdnonfilt10%	0.8*	6* (7●)	15* (7.2●)	1.4* (3●)	0.6*	2*	12* (7.5●)
3rd dig 1%	0.8*	1.7*	1.5*	4* (6●)	22*	0.01*	0.01*
water	3.5*	4.1*	4.3*	2.3*	1.3*	1.5*	0.5*

** = highest IF in sample, ● = time in hours when the inhibitory activity begins. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex*

Figure 32 declares that filtrated 10% sample of the 1st slurry clearly stands out of other ones due to its high response. It can be if the dissolved particles in the slurry matrix provide better conditions for the signal formation. But, on the other hand, the nonfiltrated 10% sample has lower signal, so the particles can compete with chemicals which decrease the response.

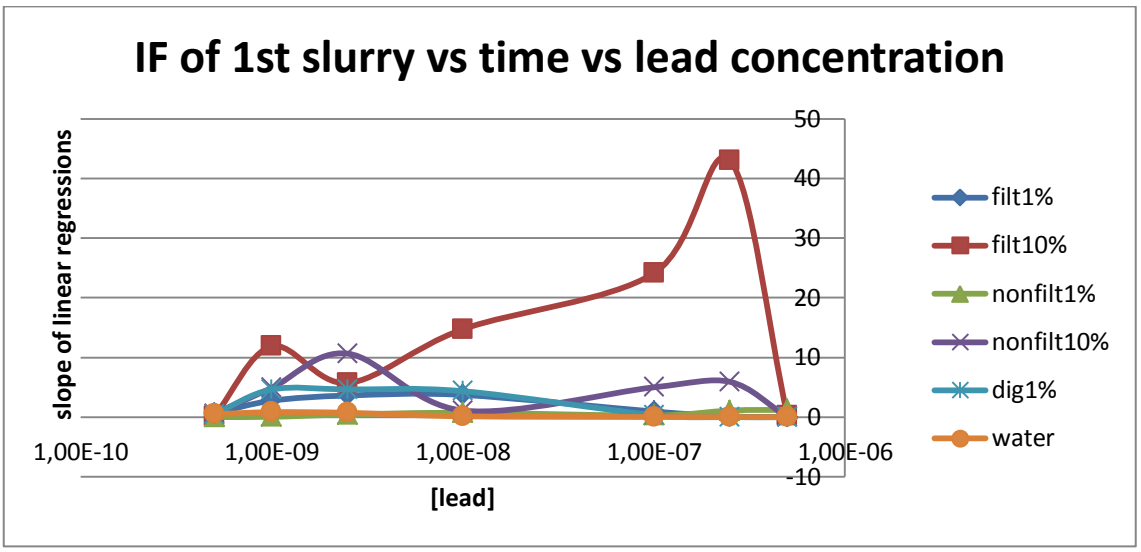

Figure 32. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs lead concentration of 1st slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

Summarizing graph (Fig. 33) indicates that the responses of the nonfiltrated samples of the 2nd slurry are very low and comparable to water. The filtrated slurries, on the other hand, have the higher response. 10% concentration of dissolved macromolecules increases activity twice. This situation occurs when the particles decrease the activity, but the dissolved media, on the opposite, supports the biosensor responses.

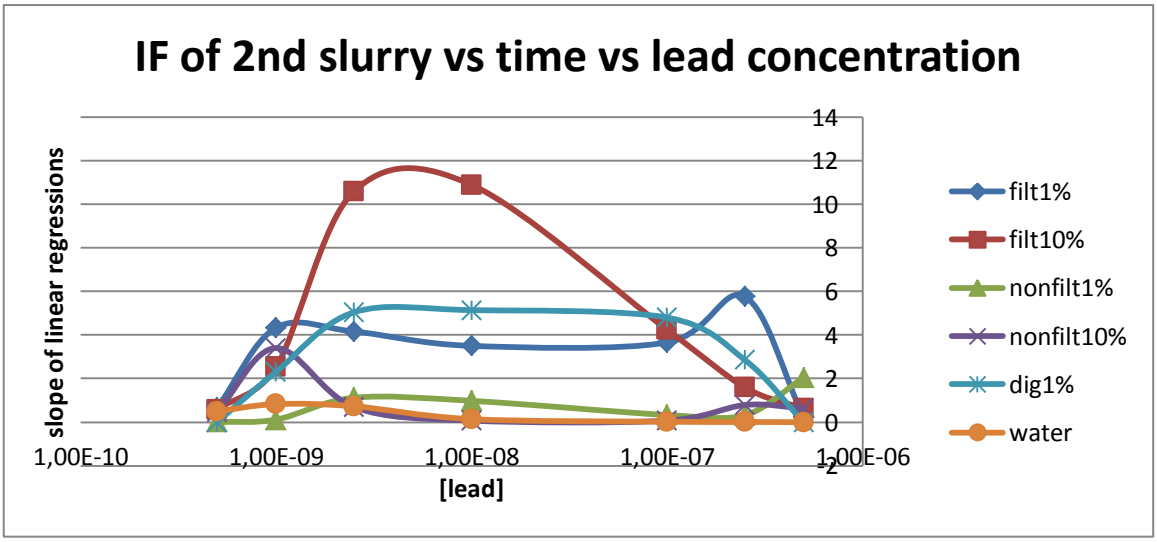

Figure 33. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs lead concentration of 2nd slurry.*
Logarithmic scale.Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

Figure 34 is a combination of the linear regression slopes of the curves of the 3rd slurry. The pattern differs to the other ones. Both 10% samples have highest sensitivity, followed by the 1%which performs the same shape of the curves as the 2nd slurry. Digested sample has clearly determined peak at 100nM point and the lowest sensitivity among the pretreatment variants .

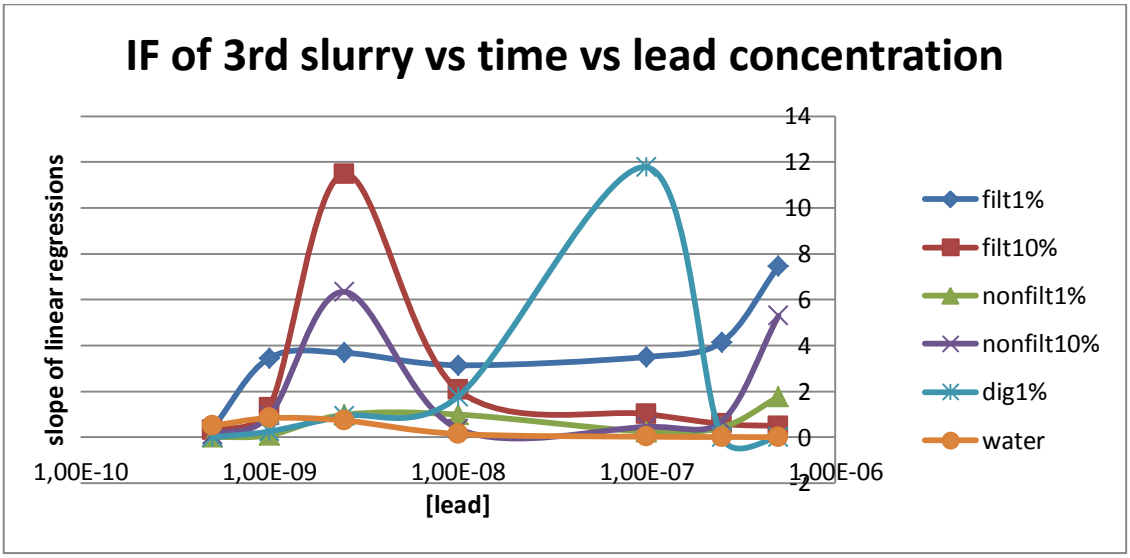

Figure 34. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs lead concentration of 3rd slurry.*
Logarithmic scale.Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

None of the pretreatment or dilution of samples do not create response that corresponds to the true amount of ions in the system and cannot be appropriate for the evaluation (Table 14). Some of them, such as the nonfiltrated samples in both dilutions are overreact with the lead addition. On the other hand, a lot of the responses exceed the water sample one; consequently, it is not possible to evaluate the lead concentration with the standard curve at all.

Table 14. *Comparison of the actual amount of lead with the amount evaluated by standard curve.*

	50pM	100pM	1nM	10nM	50nM	100nM	500nM
1st filt 1%	1nM	n/d	n/d	n/d	2.5nM	25nM	500nM
1stfilt10%	<100pM	n/d	n/d	n/d	n/d	n/d	100nM
1stnonfil1%	100pM	100nM	500nM	>500nM	>500nM	>500nM	>500nM
1stnonfilt10%	25nM	n/d	n/d	100nM	500nM	500nM	>500nM
1st dig 1%	<50pM	<50pM	n/d	n/d	15nM	500nM	500nM
2nd filt 1%	<50pM	n/d	n/d	n/d	n/d	n/d	2.5nM
2nd filt10%	<50pM	75pM	100pM	1nM	1.25nM	2.5nM	5nM
2nd nonfil1%	100pM	100nM	500nM	>500nM	>500nM	>500nM	>500nM
2ndnonfil10%	25nM	50nM	50nM	500nM	>500nM	>500nM	>500nM
2nddig 1%	<50pM	50pM	n/d	n/d	n/d	n/d	100nM
3rd filt 1%	<50pM	100pM	n/d	n/d	n/d	n/d	n/d
3rd filt10%	50pM	75pM	1nM	2.5nM	5nM	7.5nM	8nM
3rd nonfil1%	100pM	100nM	500nM	>500nM	>500nM	>500nM	>500nM
3rdnonfil10%	25nM	50nM	n/d	100nM	>500nM	>500nM	>500nM
3rd dig 1%	<50pM	<50pM	<50pM	50pM	n/d	50nM	100nM

n/d indicates the response higher than the level of the blank result.

Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex .

4.6.4 Zinc addition

Zinc is measured with the same sensor as lead; but the response is significantly higher for all the samples. The behavior of the sensor in the zinc addition is shown on the example of the 1st slurry in the 10nM addition (Fig. 35). The 1% filtrated sample reaches the inhibitory concentration and its response is IF 215 at 108 min as maximum. Nonfiltrated 1% sample, on the other hand, has rather small response IF 29 while the digested 1% and filtrated 10% are about IF 100.

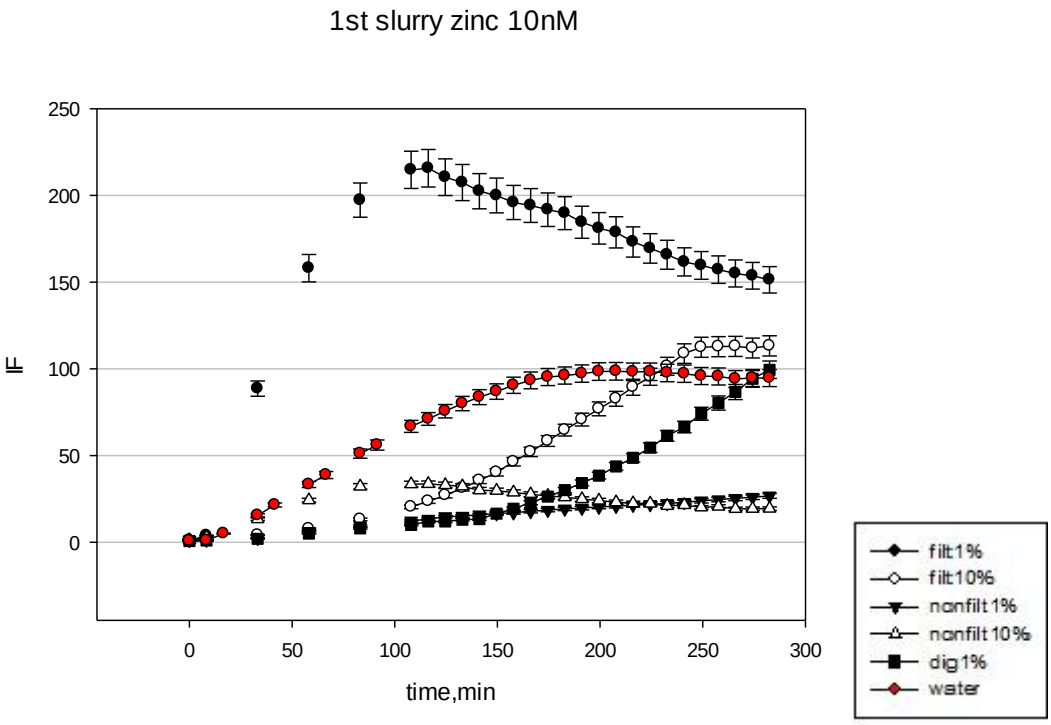

Figure 35. *Dynamics of 1st slurry nonfiltrated 1% and 10%, filtrated 1% and 10%, digested 1% with 10nM ZnCl2 added.*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

Zinc stimulates the cells emit more light or let more cells survive (Table 15). Increasing of zinc concentration to some point possibly can even stimulate the cells in case of deficiency. The digested sample works better on lower concentrations but soon silence down; in case of medium to high concentration there is a long lag phase performed. Both particles and dissolved compounds protect the cells in medium concentrations and so the signal is very high. It can be due to the aggregation of the ions on the particle surfaces and in the macromolecules. But on the other hand, the combination of both the properties, on the opposite, decreases the signal.

Table 15. *Summary of the slurries behavior with zinc addition*

	500pM	1nM	10nM	50nM	100nM	500nM	1µM
1st filt 1%	4.5*	38.5*	215* (108●)	81*	17*	1.8*	0.3*
1st filt10%	1.9*	16*	109*	148*	262*	2*	0.5*
1st nonfil1%	1.7*	3*	40* (82●)	201*	119*	16.2*	1.2*
1stnonfilt10%	2*	4*	29*	269* (125●)	104* (108●)	13.8* (108●)	1.2* (108●)
1st dig 1%	40*	46*	100*	88*	3*	0.1*	0.1*
2nd filt 1%	6.7*	60*	189* (158●)	21*	1*	3*	0.6*
2nd filt10%	3.1*	38*	293*	183*	28*	1*	0.5*
2nd nonfil1%	1.5*	2.5*	61*	494*	146*	60*	1.4*
2ndnonfilt10%	1.4*	2.3*	22*	56*	253*	6*	5*
2nddig 1%	7.8*	19*	196*	152*	1*	1.2*	0.2*
3rd filt 1%	11.7*	96*	130* (98●)	35* (150●)	1.2*	1*	0.3*
3rd filt10%	2*	9.6*	46*	179*	42*	4*	0.9*
3rd nonfil1%	3.6*	57*	473*	163*	162*	18*	1.6*
3rdnonfilt10%	3.8*	7.2*	11*	41* (108●)	28* (134●)	63* (134●)	7.6* (134●)
3rd dig 1%	1.2*	3*	6*	24*	12*	1.8*	0.2*
water	2.2*	20*	101*	78*	23*	1.2*	0.5*

** = highest IF in sample, ● = time in hours when the inhibitory activity begins. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 4 hours incubation at 30°C in Hidex*

Figure 36 shows that the response of the digested 1% sample resembles the water one most of all. The filtrated samples are more sensitive and filtrated 1% has the higher response due to less concentration of the dissolved chemicals. The nonfiltrated samples, on the opposite, have the higher response with higher concentration due to possible particle protection.

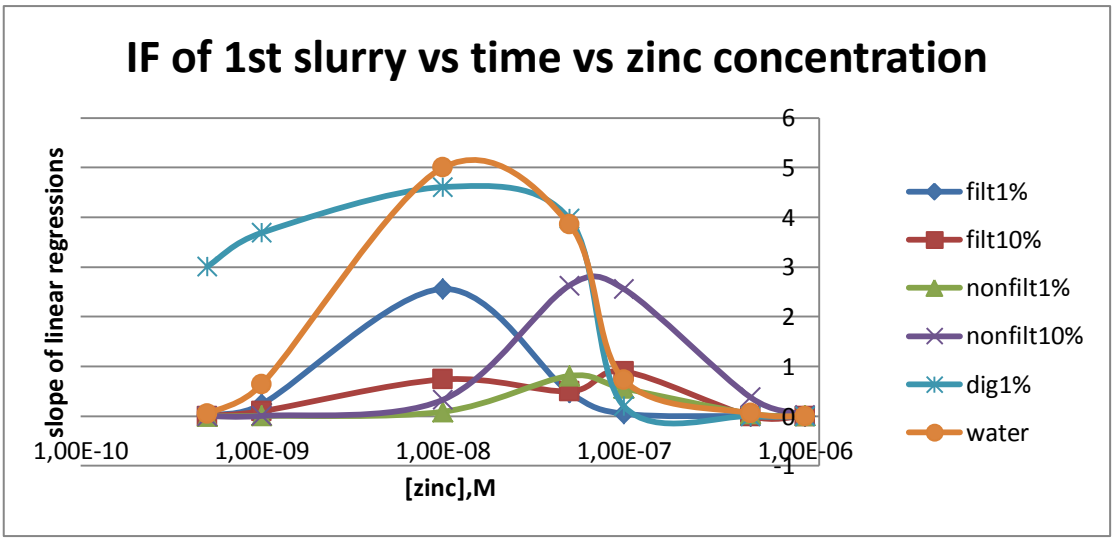

Figure 36. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs zinc concentration of 1st slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 4 hours incubation at 30°C in Hidex

In case of the 2nd slurry (Fig. 37), the response of the digested sample exceeds the water. The difference in percentage does not interfere so much to the responses, unlike of the 1st slurry – the filtrated samples have almost overlapping curves and the nonfiltrated ones are very close too. The sensitivity of the nonfiltrated 10% sample decreases from 50 µM to 100µM.

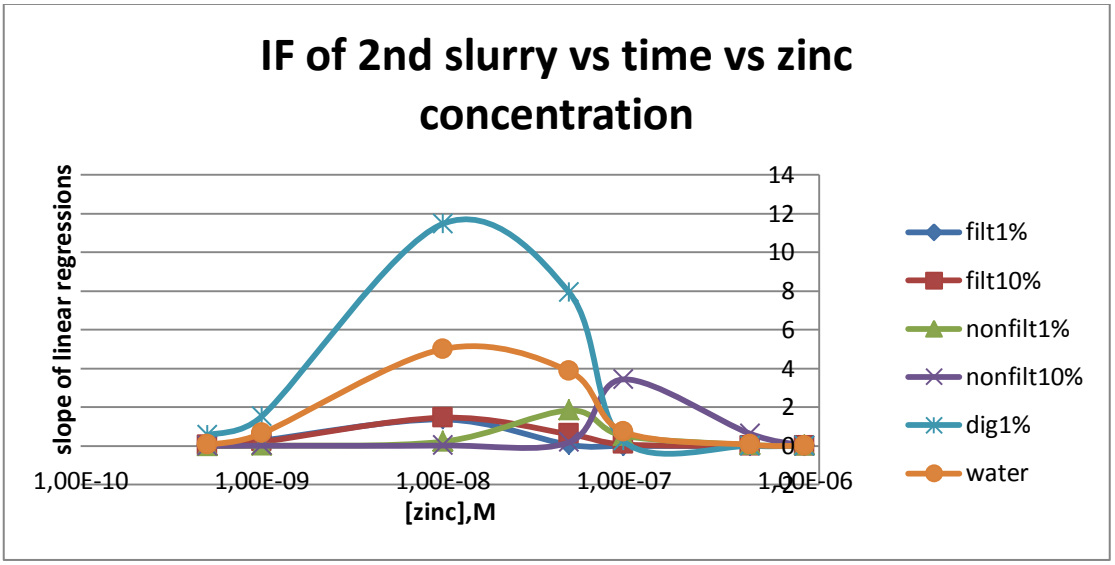

Figure 37. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs zinc concentration of 2nd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 4 hours incubation at 30°C in Hidex

The digested 1% sample has less activity at low concentrations (Fig. 38). The filtrated 1% has the highest sensitivity but its response is not so significant. Then the nonfiltrated 1% and filtrated 10% follow, and then the nonfiltrated10%. So there can be an output that the particles decrease the response. But because the digested sample has high response as well, the reason can be not due to an aggregation of zinc, but in ability to release extra bivalent ions.

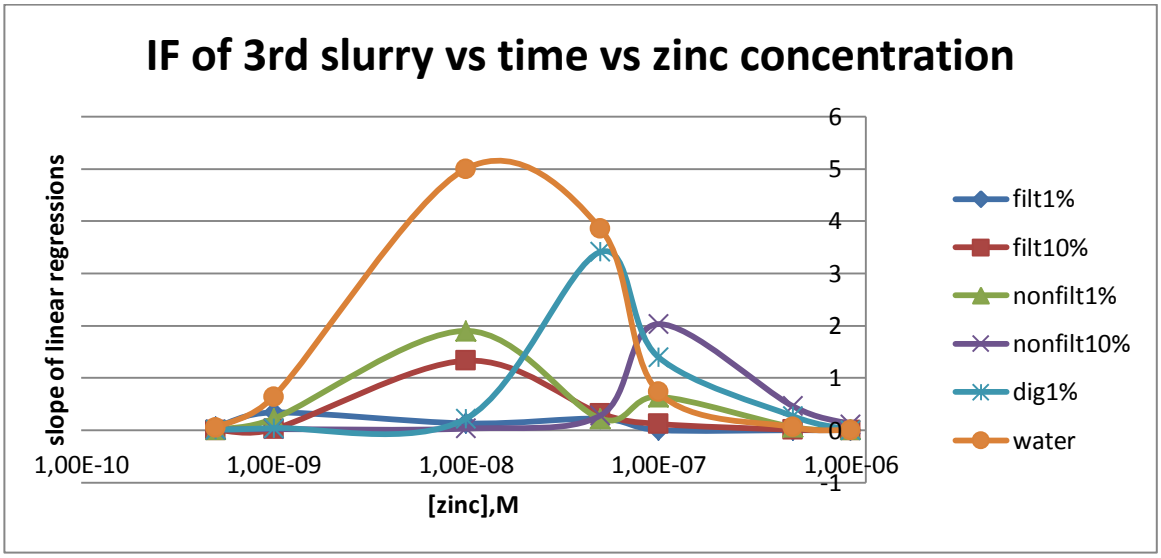

Figure 38. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs zinc concentration of 3rd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 4 hours incubation at 30°C in Hidex

According to summarizing table 16, that represents the comparison of the amount of ions added and the amount evaluated by obtained response, the best choice for the zinc assay would be filtrated 10% slurry. The filtrated 1% and the nonfiltrated 1% samples also give quite reliable data. Nonfiltrated 10% decrease the signal with retaining the most part of the ions. Digested 1% sample can give very precise response, for instance, the 2nd slurry but in case of the 1st and the 3rd slurry it shows data poorly comparable to the original one.

Table 16. *Comparison of the actual amount of zinc with the amount evaluated by standard curve.*

	500pM	1nM	10nM	50nM	100nM	500nM	1μM
1st filt 1%	600pM	1.25nM	n/d	20nM	80nM	500nM	1μM
1st filt10%	500pM	1nM	10nM	n/d	100nM	500nM	1μM
1stnonfilt1%	500pM	750pM	15nM	n/d	115nM	750nM	1μM
1st nonfil10%	n/d	n/d	15nM	n/d	n/d	75nM	500nM
1st dig 1%	12nM	15nM	40nM/ 75nM*	80nM	100nM	500nM	1μM
2nd filt 1%	750pM	2.5nM	n/d	80nM	500nM	600nM	1μM
2nd filt10%	750pM	1.25nM	n/d	n/d	90nM	500nM	1μM
2nd nonfil1%	500pM	1nM	25nM	n/d	n/d	800nM	1μM
2ndnonfilt10%	n/d	n/d	500pM	n/d	n/d	n/d	222nM
2nddig 1%	500pM	1nM	45nM	60nM	100nM	500nM	1μM
3rd filt 1%	750pM	10nM	10nM	75nM	500nM	750nM	>1μM
3rd filt10%	<500pM	750pM	1.25nM	n/d	90nM	500nM	750nM
3rd nonfil1%	500pM	1nM	25nM	n/d	n/d	750nM	1μM
3rdnonfilt10%	<500pM	500pM	750pM	2.5nM	n/d	n/d	110nM
3rd dig 1%	<500pM	<500pM	<500pM	10nM	100nM	250nM	500nM

n/d indicates the response higher than the level of the blank result. indicates presence of two values on the standard curve that subjected to the obtained response*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 4 hours incubation at 30°C in Hidex .

4.6.5 Cadmium addition

Cadmium is a metal that the biosensors were originally adapted for. But the response is quite low due to not perfectly adapted pH conditions – the biosensors work better at 7.0, but still give some response at 6.9, which was chosen for this study. On the other hand, detection limit is higher: not nanomolar concentrations but micromolar allow to be tested.

Concentrations higher than 100μM are extremely toxic for the samples. The filtrated 10% and the nonfiltrated 10% samples have the highest responses. Almost all samples perform the inhibitory activity at 10-50μM.

Filtrated 10% of the 3rd slurry shows an interesting behavior at 50 μM – after the clearly inhibitory rising and abrupt drop, it repeats a growth after phase of stability. It can be if the cells adapt to the condition and can be an evidence of response due to increasing of cell number.

Table 17. *Summary of the slurries behavior with cadmium addition*

	100nM	1µM	5 µM	10 µM	50 µM	100 µM	250 µM
1st filt 1%	1.4*	9*	15*	5*	1*	0.5*	0.1*
1st filt10%	2.1*	5.5*	200*	136* (270●)	1*	0.4*	0.1*
1st nonfil1%	1.5*	4.1*	10*	4*	1.8*	0.9*	0.1*
1stnonfilt10%	1.8*	15*	42* (202●)	37* (180●)	1*	0.5*	0.1*
1st dig 1%	1.8*	3*	15*	6*	6.6* (120●)	0.8*	0.1*
2nd filt 1%	1.8*	7*	18*	5.5*	1*	0.2*	0.1*
2nd filt10%	12.1*	11*	30*	28*	4*	0.4*	0.2*
2nd nonfil1%	1.7*	3*	3*	2*	1.9* (202●)	0.4*	0.2*
2ndnonfilt10%	1.5*	5*	5*	2* (196●)	1.5* (120●)	0.8*	0.4*
2nddig 1%	2*	3*	15*	20*	1.8* (121●)	0.8*	0.3*
3rd filt 1%	0.9*	5.4*	15*	5.3* (209●)	0.2*	0.1*	0.1*
3rd filt10%	4*	5.6*	25*	15.3* (180●)	9.9* (108●)	3*	0.1*
3rd nonfil1%	0.3*	2.5*	4.8*	3.7*	2.1*	0.1*	0.1*
3rdnonfilt10%	0.3*	4.9*	25*	2.1* (202●)	3.1* (98●)	0.1*	0.1*
3rd dig 1%	0.2*	1.5*	7.1*	14.1*	3.8*	0.5*	0.2*
water	0.1*	2*	7.1*	8.2*	3.6*	0.5*	0.1*

** = highest IF in sample, ● = time in hours when the inhibitory activity begins. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex*

The summary graph of the 1st slurry (Fig. 39) shows that filtrated 10% is the only sample which exceeds the water response and the nonfiltrated 10% sample also has a high value but not enough to overpass the water. The digested 1% has an extra signal rising at 250µM, when other concentrations show no signals; it can be an artifact piece of data.

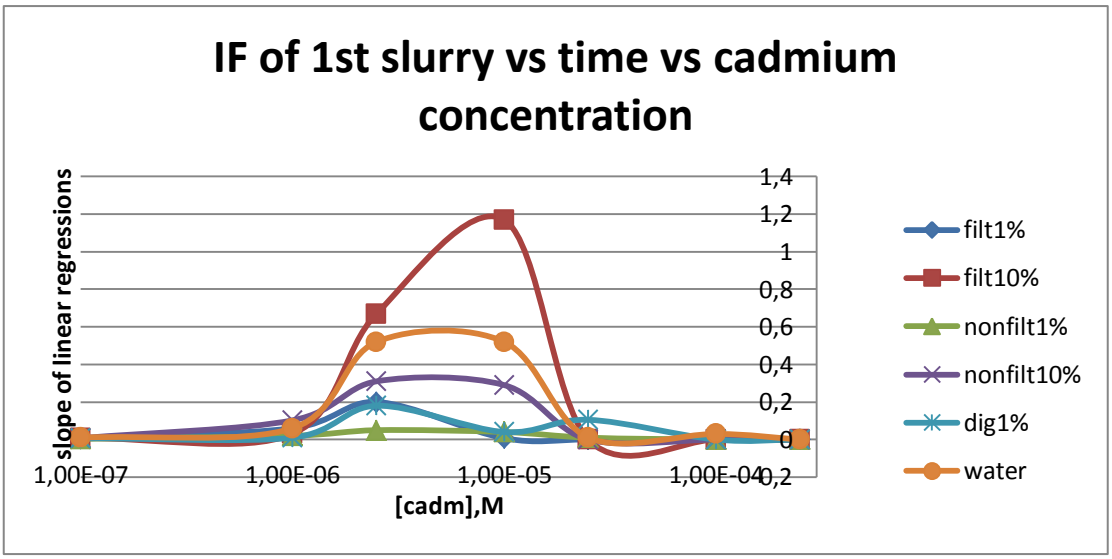

Figure 39. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs cadmium concentration of 1st slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

The combination of all the curves of the 2nd slurry on the figure 40 indicates that the slurry suppress the signal formation. There can be two tendencies found, that the particles suppress the ion providing to the biosensors and so decrease the response – the nonfiltrated samples have lower signals. On the other hand, macromolecules facilitate the signal – the 10% concentration increases the response.

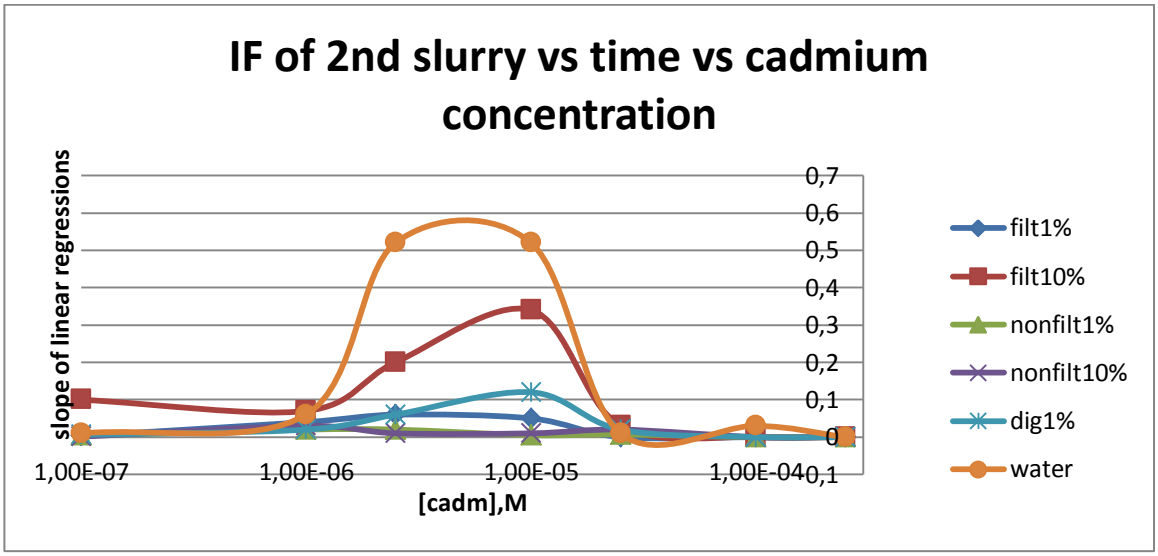

Figure 40. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs cadmium concentration of 2nd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

The summary of the 3rd slurry activity (Fig. 41) shows that the cells perform less response than water in all cases and the filtrated 10% has steady response in broad range. The nonfiltrated 10% and the filtrated 1% have higher sensitivity. Digested sample works on higher concentrations but its response level is comparable to the filtrated 10%. Nonfiltrated 1% has the lowest signal among all.

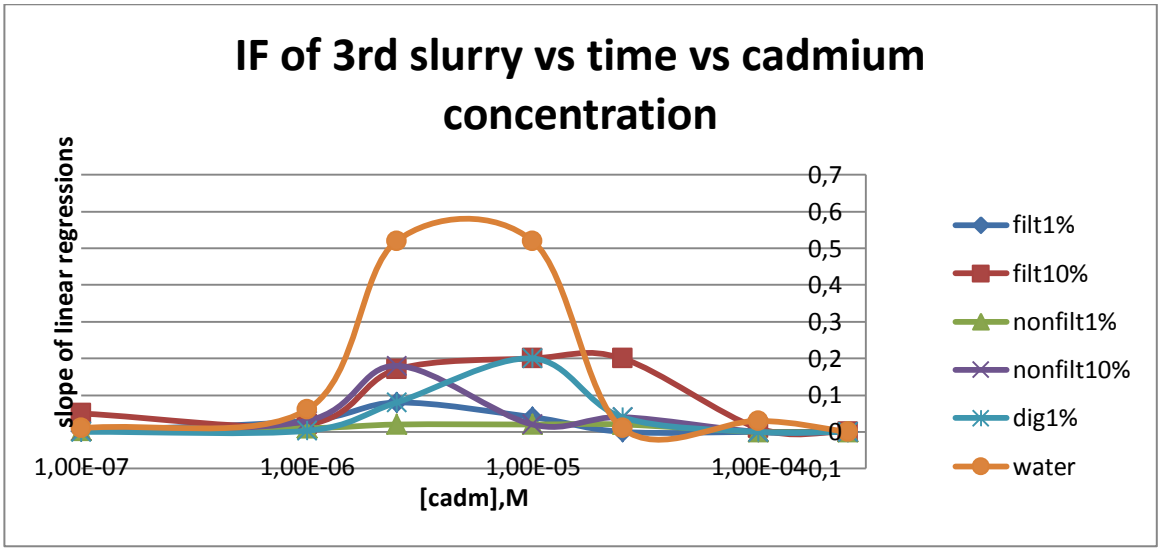

Figure 41. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs cadmium concentration of 3rd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex

Filtrated 1% could be the best choice for the determination of the cadmium amount in samples (Table 18). Nonfiltrated 1% provides reliable information as well. But the responses of other samples do not reflect the actual ionic composition.

Table 18. *Comparison of the actual amount of cadmium with the amount evaluated by standard curve.*

	100nM	1µM	5µM	10µM	50µM	100µM	250µM
1st filt 1%	100nM	2.5µM	5µM	25 µM	75 µM	<250µM	>250µM
1st filt10%	<100nM	800nM	n/d	n/d	100 µM	250 µM	>250µM
1stnonfilt1%	750nM	2.5 µM	5 µM	15 µM	50 µM	250µM	>250µM
1st nonfil10%	500nM	n/d	n/d	25 µM	>250µM	>250µM	>250µM
1st dig 1%	<100nM	<100nM	100nM	30 µM	50 µM	250µM	>250µM
2nd filt 1%	100nM	1 µM	5 µM	10 µM	50 µM	100 µM	250µM
2nd filt10%	<100nM	1.1 µM	1.5 µM	75 µM	75 µM	250µM	>250µM
2nd nonfil1%	200nM	1.1 µM	25 µM	50 µM	50 µM	250µM	>250µM
2ndnonfilt10%	100nM	250nM	7.5 µM	40 µM	>250µM	>250µM	>250µM
2nddig 1%	<100nM	<100nM	100nM	n/d	80 µM	>250µM	>250µM
3rd filt 1%	110nM	1.25 µM	2 µM	11 µM	50 µM	>250µM	>250µM
3rd filt10%	<100nM	900nM	1.25 µM	75 µM	75 µM	75 µM	250µM
3rd nonfil1%	200nM	1.2 µM	1.5 µM	25 µM	45 µM	250µM	>250µM
3rdnonfilt10%	100nM	250 nM	n/d	25 µM	75 µM	>250µM	>250µM
3rd dig 1%	<100nM	<100nM	<100nM	100nM	50 µM	80 µM	90 µM

n/d indicates the response higher than the level of the blank result.
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 8 hours incubation at 30°C in Hidex .

4.6.6 Nickel addition

The nickel tests have the longest duration because not all of them answer the addition in the same time. Furthermore, part of the experiment is taken not in a luminescence counter but in a shaker, due to low results in the beginning and so to reduce time of the machine work. Also the nonfiltrated 10% samples of the 2nd and the 3rd slurries showed no response so the percentage was decreased to 5%.

The behavior of the slurry activity with nickel added is showed on the 2nd slurry with 1nM nickel added (Fig. 42). It is toxic for the filtrated1% sample so the response abruptly falls from IF 4.1 to IF 2 at 12th hour of the experiment. Nonfiltrated 5% has a short-term rising from IF 2 to IF 4 at 10 hour point. Water sample has two peaks – first one at 11.5 hours and the sample reach IF 2, and the second with IF 10.8 in the end of measurement. But the same concentration is not enough for other samples to react, so their responses stay low.

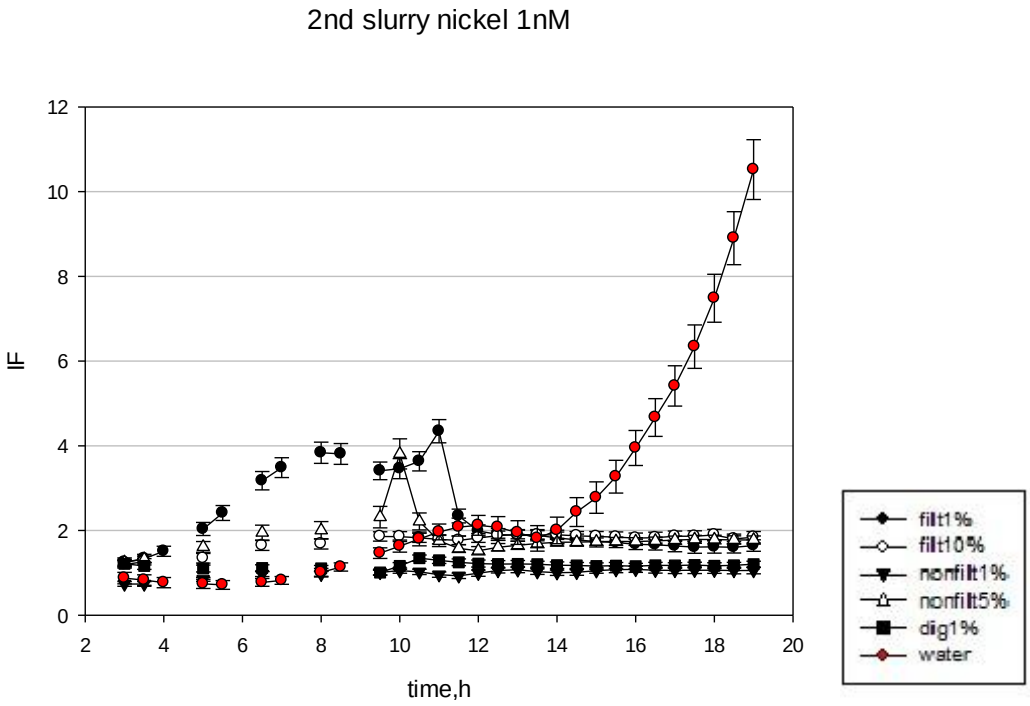

Figure 42. *Dynamics of 2nd slurry nonfiltrated 1% and 5%, filtrated 1% and 10%, digested 1% with 1nM NiSO4 added.*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex

With nickel added the signal grows after 8-12 hours of incubation. This may occur due to cell adaptation, and so the response is not only because of the gene activity but also because of increasing of cell number. The nonfiltrated samples have higher maximum responses and the high-percented have higher than 1% (Table 19). This can be if the particles protect the biosensors in high concentration, but on the other hand, if the dissolved material provides better condition for the signal formation.

Table 19. *Summary of the slurries behavior with nickel addition.*

	100pM	250pM	500pM	1nM	5nM	10nM	50nM
1st filt 1%	1*	1.1*	2.5* (9●)	120* (15●)	2* (8●)	54* (4.5●)	10*
1st filt10%	1.2*	5.7* (5●)	6* (6●)	120* (5●)	4.8* (4.5●)	50* (8●)	8*
1st nonfil1%	1.1*	1.3*	2* (14●)	6* (13●)	2* (10●)	64*	175*
1stnonfilt10%	1.3*	2.7* (6●)	2.6* (6●)	4*	5.2* (6.5●)	265*	56* (5●)
1st dig 1%	1.3*	2*	3.1* (10●)	57* (12●)	2.8* (10●)	1*	0.9*
2nd filt 1%	0.8*	1*	2.8* (8●)	4* (11●)	2*	0.5*	0.2*
2nd filt10%	0.9*	2.5* (6●)	1.4*	2*	5*	8*	0.9*
2nd nonfil 1%	0.9*	0.8*	1*	1.5*	11*	45*	0.2*
2ndnonfilt 5%	1.1*	1.3* (8●)	2.5* (10●)	4* (11●)	3*	60* (16●)	0.1*
2nddig 1%	1.2*	1.4* (8●)	1.3* (10●)	1.5*	0.2*	55*	0.2*
3rd filt 1%	1.1*	1.7* (10●)	82* (11.5●)	2* (10●)	7*	30*	0.5*
3rd filt10%	1.2*	1.5* (9.5●)	2.5*	1.2*	10*	10*	3* (12●)
3rd nonfil 1%	2.2* (7●)	2.3* (9.5●)	1.5*	2.6* (14●)	11*	292*	0.8*
3rdnonfilt 5%	0.9*	2* (13.5●)	2*	2.1* (8.5●)	96*	391* (12●)	2*
3rd dig 1%	1*	1*	2.3*	1.5*	5*	25*	23*
water	3.2*	3.4*	22*	10.6*	6*	5*	0.9*

** = highest IF in sample, ● = time in hours when the inhibitory activity begins.*
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex

Combination of all the linear regressions of the curves (Fig.43) indicates that all the samples have increasing of the response at 500pM concentration, but the nonfiltrated ones and the filtated10% have extra peak at 5-10 nM. One explanation that there are extra ions, like zinc, in the particles and trapped in the macromolecules those are released after addition of the nickel with exchange mechanism and create extra signal.

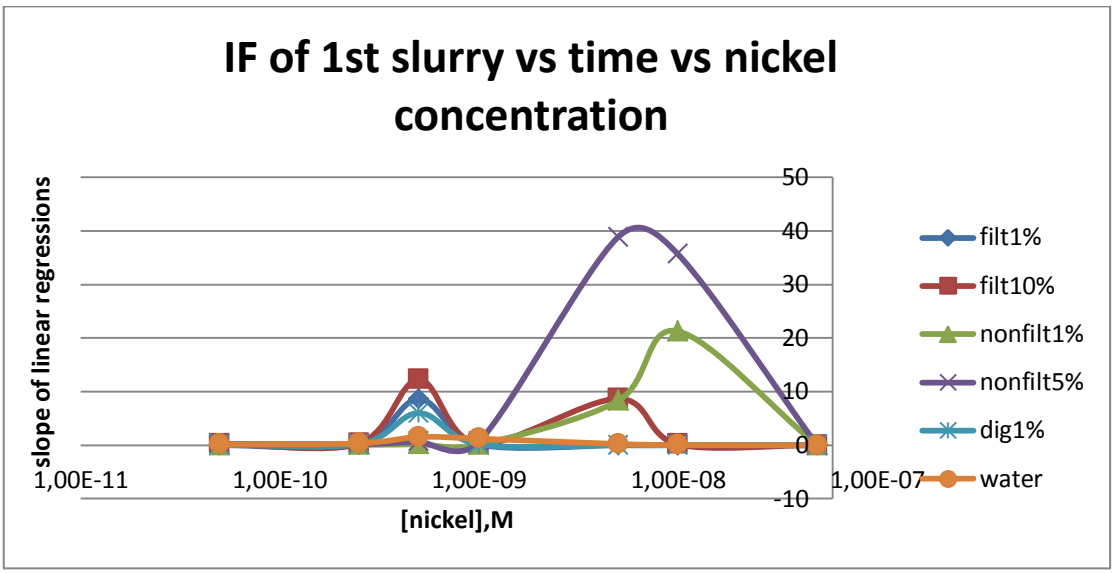

Figure 43. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs nickel concentration of 1st slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex

There are two peaks seen on Fig. 44 – one of water and filtrated samples with 500pM nickel added as highest response. The nonfiltrated and the digested samples perform a hump at 10nM. It can be due to extra bivalent ions in particles that release after nickel addition in exchange mechanism. Digested sample contains these ions already extracted.

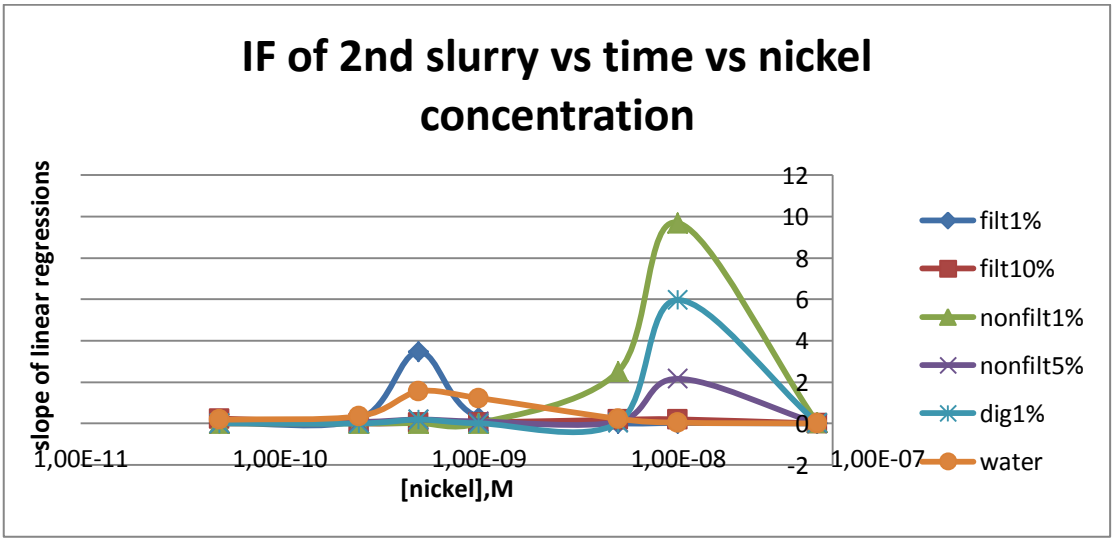

Figure 44. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs nickel concentration of 2nd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex

Figure 45 of the linear regression slopes of the 3rd slurry indicates that the filtrated samples differ in sensitivity – the filtrated 1% preferably reacts with lower concentration, such as 500pM, in comparison to filtrated 10% which works at 10nM. Nonfiltrated samples work in the same concentrations of nickel – the 5% performs better response that the nonfiltrated 1%. Digested 1% sample also has two peaks – at 10nM and the second one at 500nM. The graph indicates that both the dissolved organic matter and the particles decrease sensitivity of the biosensors.

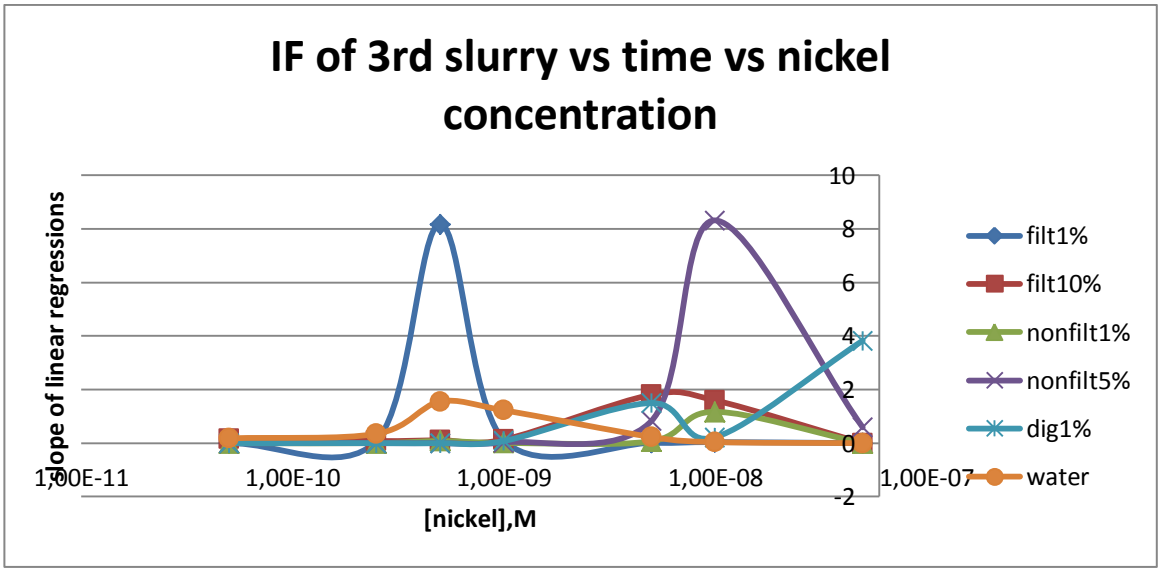

Figure 45. *Combination of linear regression slopes of all the dynamic curves. IF vs time vs nickel concentration of 3rd slurry.*
Logarithmic scale. Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex

To evaluate the nickel concentration (Table 20) digested samples for the 1st slurry can be the best. But the nonfiltrated 5% allows getting precise results in the 2nd and the 3rd slurries. This table also supports that the 1st slurry differs from the 2nd and the 3rd ones in case of added nickel. The rest variants of treatment do not reflect the actual concentration added.

Table 20. *Comparison of the actual amount of nickel with the amount evaluated by standard curve.*

	50pM	100pM	500pM	1nM	5nM	10nM	50nM
1st filt 1%	<50pM	<50pM	n/d	50nM	50nM	50nM	>50nM
1st filt10%	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM
1stnonfilt1%	100pM	100pM	1nM	1nM	n/d	n/d	45nM
1st nonfil10%	100pM	100pM	500pM	2.5nM	25nM	40nM	n/d
1st dig 1%	<50pM	<50pM	500pM	1nM	5nM	10nM	>50nM
2nd filt 1%	<50pM	<50pM	1nM	7.5nM	50nM	50nM	>50nM
2nd filt10%	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM
2nd nonfil1%	50pM	50pM	500pM	500pM	n/d	n/d	45nM
2ndnonfilt5%	50pM	100pM	500pM	800pM	1nM	10nM	45nM
2nddig 1%	<50pM	<50pM	<50pM	<50pM	<50pM	n/d	50nM
3rd filt 1%	<50pM	<50pM	750pM	7.5nM	50nM	50nM	>50nM
3rd filt10%	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM	<50pM
3rd nonfil1%	100pM	500pM	500pM	1nM	n/d	n/d	45nM
3rdnonfilt5%	50pM	100pM	100pM	100pM	5nM	10nM	45nM
3rd dig 1%	<50pM	<50pM	<50pM	<50pM	500pM	1nM	10nM

n/d indicates the response higher than the level of the blank result.
Operation conditions: Pseudomonas putida K2431.2440 pDNPczclux1 after 19 hours incubation at 30°C in Hidex .

5 Conclusions

According to the data, obtained in this study, dissolved material in slurry can both facilitate and impede the cell reaction. There are several possible reasons - the cells can grow slower or faster in the matrix due to high nutrient content or, on the other hand, the dissolved macromolecules can prevent ion penetration through the cell wall.

There also can be some ions attached to the particles, so after addition of other metals an exchange mechanism occurs, and this trapped fraction releases to the media and becomes detectable. It is a possible explanation of two humps in nickel curves.

There is also a presence of a lag-phase in the beginning – it is a time needed for penetration of ions through the cell wall and also a time for adaptation of the cells to the complicated conditions.

The choice of the sample concentration and pretreatment depends on a question faced – the minoric pretreatment reflects how much metal can reach the cells in normal conditions of utilization, while the digestion protocol can tell how much ions are actually in the system. In the first case, nonfiltrated 1% is the best variant – the dissolved molecules do not suppress the cell growth and the metal bonded in particles can be partially estimated. On the other hand, digested 1% indicates all ions that can release out of the sample during degradation process.

Feasibility of the biosensors in testing of the slurries has some serious reasons. First, the cellular biosensors have lower detection limit than physical methods, additionally, the biosensors check only bioavailable metals. Another advantage is the easiness of the measurement – no trained personal required and with an invention of a field luminometer, the testing can be made in any condition and place. The volume required for the biosensor tests is very small; therefore, it allows minimizing costs and storage place and can be potentially developed to a high-throughput methodic.

Some cells react faster on some metals and if the duration of the reaction is less than 4 hours, time is also an advantage. But the nickel and the lead tests require much more time than standard AAS protocol, so the sensing system should be adapted to these metals. Long time of measurement also decrease the reliability of the data, because after an adaptation period, the cell number and so the signal intensity increase, which do not reflect the actual metal concentrations.

Another problem is that the cells can measure several heavy metals at one moment. So in complex matrices the sensors can give a total feedback. To obtain more accurate data the standard addition method should be involved. The last, but not the least, problem is that the cells, in some cases, work in a quite narrow range of pH. So the samples should be adjusted prior the measurement directly for the particular biosensor requirements.

Whole-cell biosensor technology opens new horizons in environmental study, so further development and research of functioning in such complex media are needed.

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